REMARKS

This is in response to the Office Action dated November 3, 2004. A request for a two month extension of time is included herewith. Claims 40, 54, 61-65, 72-75, 82-86, 93 and 94 are currently pending in the application. Claims 40, 54, 61-65, 72-75, 82-86, 93 and 94 are amended herein.

Applicants note with appreciation that the Examiner has withdrawn rejections of claims 40, 74 and 84 under 35 U.S.C. §112, second paragraph. Applicants also gratefully acknowledge withdrawal of the rejection of claims 40, 54, 61-65, 72-75, 82-86, 93 and 94 under 35 U.S.C. §112 as containing new matter. Applicants note withdrawal of the rejection of claims 40, 54, 61-65, 72-75, 82-86, 93 and 94 under 35 U.S.C. §112, first paragraph as containing an inadequate written description.

Applicants also note that the rejection of claims 40, 54, 61-65, 72-75, 82-86, 93 and 94 under 35 U.S.C. §102 over references to Cover *et al.* have been withdrawn, but modified to meet the limitations of the claims. The new rejections are addressed herein.

Finally, Applicants confirm that a terminal disclaimer over U.S. Serial No. 09/921,157 will be submitted upon receipt of an indication of allowability.

Claim Amendments

Applicants herein amend claims 61, 62, 72, 73, 82, 83, 93 and 94 to include the feature that when the recombinant polypeptide is at least 87 or 100 kDa, the polypeptide comprises amino acid substitutions to form an altered polypeptide, wherein the altered polypeptide exhibits no cytotoxic activity or reduced cytotoxic activity compared with a native *Helicobacter pylori* cytotoxin. These claims have been further amended to place an upper limit on the size of the protein at about 140 kDa. Support for the amendment may be found, for example, at page 8, lines 20-23 for exhibiting no cytotoxic activity or reduced cytotoxic activity, and at page 5, lines 31-35 for the upper limit of the protein size (*i.e.*, full-length cytotoxin precursor). Claims 40, 54, 61-65, 72-75, 82-86, 93 and 94 have been further amended to include the feature that the polypeptide is purified. Support for the amendment may be found, for example at page 16, lines 19-29. No new matter is added.

Summary

Upon review of the instant Office Action, it is apparent that a review of the overall technology may be helpful in understanding the scope of the claimed invention. The CT Toxin of *Helicobacter pylori* was identified as an 87 kDa protein that has cytotoxic activity. That is, the protein caused vacuolization and cell death. However, the Applicants have discovered that the cytotoxic protein is actually a 100 kDa protein with cytotoxic activity and the 87 kDa protein identified was probably a degraded fragment of the 100 kDa protein or a further processed fragment of the 100 kDa polypeptide. Applicants have discovered that the full-length precursor protein is actually approximately 140 kDa. This 140 kDa protein has the amino acid sequence of SEQ ID NO:3 and requires proteolytic processing to generate the cytotoxic protein. Thus, the polypeptide having the amino acid sequence of SEQ ID NO:3 ***is not itself cytotoxic***. The Applicants have also described that the full-length precursor protein (SEQ ID NO:3) may be used as an immunogen to generate antibodies that also recognize the cytotoxic protein. In addition, protein fragments may also be used to generate antibodies that bind the cytotoxic protein, but which peptides are not cytotoxic themselves. Clearly, the goal is to administer a non-cytotoxic form of the protein (either a precursor or non-cytotoxic fragments) so antibodies could be generated without causing an untoward effect in the subject.

The claims are drawn to reflect that the isolated polypeptides used in the immunogenic compositions are either the precursor or non-cytotoxic fragments, and are "immunologically identifiable" with the cytotoxic form of the protein. With this overall view in mind, Applicants address the Office Action as follows.

New Matter

The Office Action maintains the rejection of claims 40, 54, 61-65, 72-75, 82-86, 93 and 94 as allegedly containing new matter. The Examiner asserts that page 5, lines 31-39 of the Specification teaches that the polypeptide having the amino acid sequence of SEQ ID NO:3 has cytotoxic activity and causes vacuolization and death of a number of eukaryotic cell types. This is not true, and is a misreading of that passage.

The Specification teaches at page 5, lines 31-39 that the term cytotoxin or toxin is a general term referring to the protein of *Helicobacter pylori*. However, this passage clearly teaches that the precursor protein (which has the amino acid sequence of SEQ ID NO:3) is a protein of 140 kDa and the proteolytic fragment has cytotoxic activity (by clear implication and generally accepted terminology in the art, the precursor does not have such activity). Further, the protein that causes vacuolization and cell death has been purified from *H. pylori* culture supernatant fluids. As described on page 6, lines 1-11, it is the processed fragment which is recovered from supernates that has cytotoxic activity and that the applicants are the ones who, for the first time, discovered that the cytotoxin is actually a processed portion of a much larger precursor protein. One of skill in the art would understand that the precursor protein (having the amino acid sequence of SEQ ID NO:3) would not be cytotoxic until processed, thus, the polypeptide of SEQ ID NO:3 would not have cytotoxic activity.

Vinion-Dubiel, A.D. *et al.* (1999) *J. Biol. Chem.* 274(53):37736-37742 ("Vinion-Dubiel") (copy enclosed for the Examiner's convenience) reported a mutant VacA cytotoxin of *H. pylori* which lacked a region of the amino terminus (Δ6-27) and not only lacked vacuolation activity, but it exerted a dominant negative effect on wild-type cytotoxin. It was later shown that the mutant lacked three tandem GXXXG motifs which were necessary for dimerization of the VacA subunits within membranes and formation of anion channels (McClain, M.S. *et al.* (2003) *J. Biol. Chem.* 278:12101-12108)(copy enclosed for the Examiner's convenience). Reyrat J.-M. *et al.* (1999) *J. Mol. Biol.* 290:459-470 ("Reyrat") (copy enclosed for the Examiner's convenience) showed that a large deletion of the cytotoxin (Δ91-330) resulted in an inactive protein. Additionally, Genisset, C. *et al.* "A *Helicobacter pylori* Vacuolating Toxin Mutant (VacA Δ49-57) Which Fails to Form Oligomeric Structures Presents a Dominant Negative Phenotype" *to be submitted* ("Genisset") (copy enclosed for the Examiner's convenience) demonstrates yet another mutant cytotoxin that has reduced or no cytotoxic activity. These investigators prove that the cytotoxin of *H. pylori* may be altered genetically to produce a cytotoxin that has substantially reduced or no cytotoxicity. This was first taught in the Applicants' specification and has now been borne out by these investigators work.

The specification also clearly teaches that a polypeptide that is "derived from" a particular nucleic acid sequence is one that has an amino acid sequence encoded by the

nucleic acid, is a portion of the encoded sequence, or is immunologically identifiable with a polypeptide encoded in the sequence. One of skill in the art would clearly understand that these are not mutually exclusive, despite the Office Action's overemphasis of the word "or." That is, one of skill in the art would appreciate that not all fragments of the encoded sequence would necessarily be immunologically identifiable with the encoded sequence. Thus, the conjunction "and" would be inappropriate to express the idea the Applicants convey in the specification. This is merely an attribute of the encoded polypeptides and certain polypeptide fragments would be immunologically identifiable with the encoded sequence. All the immunologically identifiable fragments would be at least 3-5, 8-10, or 11-15 amino acids in length. How could it be otherwise?

Finally, the Examiner is not persuaded that the original claim 8 supports the subject matter that the polypeptides have the two functional aspects of being immunologically identifiable with antibodies that react specifically with the full-length protein (SEQ ID NO:3) and which have no or reduced cytotoxicity. This is puzzling. Original Claim 8 depends from original claim 2 or 3 which claim a polypeptide that is the cytotoxin, the precursor of the cytotoxin, a derivative of the cytotoxin, or fragment of the cytotoxin. Claim 8 adds the limitation that the polypeptides have no toxicity or substantially reduced cytotoxicity. Therefore, the claims, as originally filed, clearly contemplated the precursor and fragments of the polypeptide having no or reduced cytotoxicity. As discussed above, the portions of the amino acid sequence were also said to include those that were immunologically identifiable with the encoded protein. Applicants earnestly and strongly urge the Examiner to reconsider her position and withdraw this ground of rejection.

U.S. Patent No. 6,054,132 to Cover *et al.* ("Cover I")

The Office action notes that the claims do not require that the polypeptides be "purified" and that the claims do not recite that the polypeptides have "substantially no cytotoxicity or substantially reduced cytotoxicity." The claims are herein amended to include these features.

The Examiner points out that the Cover I specification states at Col. 2, lines 40-44 that "one such embodiment is antigenic fragments of the CB antigen." The Examiner further states that "the term CB antigen includes 'antigenic fragments of the holotoxin, whether

derived from *H. pylori* or synthetically or recombinantly produced.'" However, the Examiner has ignored the full description of the term "CB antigen." Cover clearly states at Col. 2, lines 37-38 (the sentence immediately preceding the passage quoted by the Examiner): "The term CB antigen is ***defined*** as the *functionally active* non-denatured vacuolating toxin" (emphasis added). Thus, antigenic fragments must also be functionally active otherwise the definition of CB antigen can mean something other than how it is specifically defined. The Examiner cannot change the Patentee's definition of CB antigen for the purposes of a rejection by reading different parts of the specification in isolation. Further, the Examiner states that one such antigenic fragment is the 23 amino acid fragment shown in Table 2. This is just plain wrong.

As explained in the previous response, the "fragment" in Table 2 is simply a sequence that is compared to a similar amino acid sequence for ion channel or transport proteins. Apparently the Examiner believes that N-terminal sequencing produces fragments of proteins. This is not so, In N-terminal sequencing of proteins, a most amino terminal amino acid is cleaved and the amino acid is identified. The cycle continues and a second amino acid is cleaved and identified. At no point was a 23 amino acid fragment produced. Therefore it was unknown whether such a fragment would be antigenic, and it is doubtful that such a fragment would be "functionally active" as a toxin so as to fall within the Patentee's definition of "CB antigen." Nowhere in the Cover I specification is it taught or suggested that the 23 amino acid N-terminus could be used as an immunogen as contemplated by the specification. Indeed, since it would very likely not be a "functionally active" fragment (frankly admitted by the Examiner), it cannot (by the patentee's own definition) be suitable for use in the invention as a CB antigen. Applicants earnestly submit that the rejection over Cover I be withdrawn.

Cover *et al.* (1992) *J. Biol. Chem.* 267:10570-10575 ("Cover II")

PCT/EP93/00472 was filed with original claim 8 which specifically states that the polypeptides of claims 1 and 2 had "no toxicity or substantially reduced toxicity. Thus, the claims as amended are fully supported by the description of the PCT/EP93/00472. Therefore, Cover II is not available as a reference under 35 U.S.C. §102(b). Withdrawal of the rejection

is respectfully requested. However, to the extent that the rejection may be applied under another section of 35 U.S.C. §102, Applicants note the following.

Cover II teaches the purification of the 87 kDa vacuolating toxin. No fragments of the toxin were produced or evaluated. The authors performed some N-terminal sequencing, however, as described above this is not the same thing as generating a polypeptide fragment. The sequence is different from a polypeptide in much the same way as a formula for a chemical on paper is different from the actual compound. Cover II simply does not teach or suggest the use of a purified, antigenic fragment of the vacuolating toxin wherein the fragments generate antibodies that also recognize the native cytotoxin and which themselves display no cytotoxicity or substantially reduced cytotoxicity. Applicants have amended the claims to highlight that the polypeptides claimed are purified and display no cytotoxicity or substantially reduced cytotoxicity.

Withdrawal of the rejection over Cover II is respectfully urged.

New Rejection under 35 U.S.C. §112, First Paragraph (New Matter)

In Paragraph 14 of the Office Action, the Examiner modified the rejection of claims 61, 62, 72, 73, 82, 83, 93 and 94 as including new matter because the specification allegedly failed to teach an 87 kDa or 100 kDa cytotoxic protein with no or substantially reduced cytotoxicity. Applicants have amended the claims to include the clarifying feature that the polypeptides contain amino acid substitutions such they have no toxicity or substantially reduced cytotoxicity. The specification teaches at page 8, lines 20-23 that the term "recombinant polynucleotide" also encompasses the situation in which *H. pylori* bacterial genome is genetically modified (*e.g.*, by mutagenesis) to produce one or more altered polypeptides.

As shown in the references Vinion-Dubiel, Reyrat, and Genisset, alterations of the toxin have been shown to have an effect on reducing toxicity of the cytotoxin protein as taught by applicants. These investigators have now demonstrated what the applicants taught in their specification with regard to altered cytotoxin.

New Rejection under 35 U.S.C. §112, First Paragraph (New Matter)

Applicants have amended claims 61, 62, 72, 73, 82, 83, 93 and 94 to include the limitation an upper limit on the size of the protein of about 140 kDa (the size of the full-length precursor). Support for the amendment may be found, for example, at page 5, lines 31-35 wherein the upper limit of the protein size (*i.e.*, full-length cytotoxin precursor) is taught. Thus, the limitation clarifies the claim and adds no new matter. Withdrawal of the rejection is respectfully requested.

New Rejection under 35 U.S.C. §112, Second Paragraph

Claims 40, 54, 61-65, 72-75, 82-86, 93 and 94 were rejected under 35 U.S.C. §112, second paragraph as vague and indefinite for failing to provide a reference point for what "reduced cytotoxicity" means. Independent claims 40, 63, 74 and 84 have been amended to include the feature that the polypeptides have reduced cytotoxicity with respect to *Helicobacter pylori* cytotoxin purified from cell cultures. This is clearly what is contemplated by the specification, and it is supported, for example, at page 5 lines 35-39. No new matter is added. Applicants urge withdrawal of the rejection.

New Rejection under 35 U.S.C. §§102(b) and (e)(2)

The Examiner states in Paragraph 19 of the Office Action that the claims are not entitled to priority benefit of PCT/EP93/00472 filed Mar. 2, 1993 or to the foreign priority document, FI92A000052, filed Mar. 2, 1992, as these failed to disclose the limitations of "no cytotoxic activity or substantially reduced cytotoxic activity" and immunological identifiable by antibodies that specifically react with the polypeptide having SEQ ID NO:3. PCT/EP93/00472 was filed with original claim 8 which depends from original claim 2 or 3 which claims a polypeptide that is the cytotoxin, the precursor of the cytotoxin, a derivative of the cytotoxin, or fragment of the cytotoxin. Claim 8 adds the limitation that the polypeptides have no toxicity or substantially reduced cytotoxicity. Therefore, the claims, as originally filed, clearly contemplated fragments of the polypeptide having no or reduced cytotoxicity.

The claims are herein amended to include these features that the polypeptides be "purified" and have substantially no cytotoxicity or substantially reduced cytotoxicity.

The Examiner points out that the Cover I specification states at Col. 2, lines 40-44 that "one such embodiment is antigenic fragments of the CB antigen." The Examiner further states that "the term CB antigen includes 'antigenic fragments of the holotoxin, whether derived from *H. pylori* or synthetically or recombinantly produced.'" However, as discussed above, the Examiner has ignored the full description of the term "CB antigen." Cover clearly states at Col. 2, lines 37-38 (the sentence immediately preceding the passage quoted by the Examiner): "The term CB antigen is ***defined*** as the *functionally active* non-denatured vacuolating toxin" (emphasis added). Thus, antigenic fragments must also be functionally active otherwise the definition of CB antigen can mean something other than how it is specifically defined. Further, the Examiner states that one such antigenic fragment is the 23 amino acid fragment shown in Table 2. As discussed above, this is incorrect.

As explained in the previous response, the "fragment" in Table 2 is simply a sequence that is compared to a similar amino acid sequence for ion channel or transport proteins. Nowhere in the Cover I specification is it taught or suggested that the 23 amino acid N-terminus could be used as an immunogen as contemplated by the specification. Indeed, since it would very likely not be a "functionally active" fragment (frankly admitted by the Examiner), it cannot (by the patentee's own definition) be suitable for use in the invention as a CB antigen. Applicants earnestly submit that the rejection over Cover I be withdrawn.

Conclusion

Applicants earnestly submit that the claims are in condition for allowance and are patentable over the art of record. Prompt allowance is respectfully requested.

Respectfully submitted,

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A Dominant Negative Mutant of *Helicobacter pylori* Vacuolating Toxin (VacA) Inhibits VacA-induced Cell Vacuolation*

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Most *Helicobacter pylori* strains secrete a toxin (VacA) that causes structural and functional alterations in epithelial cells and is thought to play an important role in the pathogenesis of *H. pylori*-associated gastroduodenal diseases. The amino acid sequence, ultrastructural morphology, and cellular effects of VacA are unrelated to those of any other known bacterial protein toxin, and the VacA mechanism of action remains poorly understood. To analyze the functional role of a unique strongly hydrophobic region near the VacA amino terminus, we constructed an *H. pylori* strain that produced a mutant VacA protein (VacA-(Δ6–27)) in which this hydrophobic segment was deleted. VacA-(Δ6–27) was secreted by *H. pylori*, oligomerized properly, and formed two-dimensional lipid-bound crystals with structural features that were indistinguishable from those of wild-type VacA. However, VacA-(Δ6–27) formed ion-conductive channels in planar lipid bilayers significantly more slowly than did wild-type VacA, and the mutant channels were less anion-selective. Mixtures of wild-type VacA and VacA-(Δ6–27) formed membrane channels with properties intermediate between those formed by either isolated species. VacA-(Δ6–27) did not exhibit any detectable defects in binding or uptake by HeLa cells, but this mutant toxin failed to induce cell vacuolation. Moreover, when an equimolar mixture of purified VacA-(Δ6–27) and purified wild-type VacA were added simultaneously to HeLa cells, the mutant toxin exhibited a dominant negative effect, completely inhibiting the vacuolating activity of wild-type VacA. A dominant negative effect also was observed when HeLa cells were co-transfected with plasmids encoding wild-type and mutant toxins. We propose a model in which the dominant negative effects of VacA-(Δ6–27) result from protein-protein interactions between the mutant and wild-type VacA proteins, thereby resulting in the formation of mixed oligomers with defective functional activity.

Helicobacter pylori are Gram-negative bacteria that persistently colonize the gastric mucosa of humans (1). Colonization of the gastric mucosa by these bacteria results in mucosal inflammation and is a risk factor for the development of peptic ulcer disease, distal gastric adenocarcinoma, and gastric lymphoma (1–4). Gastric adenocarcinoma is currently one of the most common causes of cancer deaths worldwide and is the only cancer that has been directly linked to a bacterial infection (3).

Most *H. pylori* strains secrete a toxin (VacA) that is unrelated to any other known bacterial protein toxin (5, 6). When VacA is incubated with epithelial cells *in vitro*, the most prominent effect is the formation of large cytoplasmic vacuoles (5). These vacuoles contain markers for both late endosomes and lysosomes and have an acidic intravacuolar pH (7–9). VacA-induced vacuoles are thought to represent novel intracellular compartments that form as a result of heterotypic fusion events (7–9). In addition to altering the morphology of cells, VacA causes multiple functional changes, including alterations in the intracellular trafficking and processing of procathepsin D and epidermal growth factor (10). When added to polarized epithelial cell monolayers, VacA induces an increase in monolayer permeability for various ions and small uncharged molecules (11). VacA also interferes with the process of antigen presentation, which may be one mechanism by which *H. pylori* resists immune clearance (12).

The *H. pylori vacA* gene is translated as a 140-kDa protoxin, which undergoes amino- and carboxyl-terminal processing to yield a mature secreted toxin of about 87 kDa (13–16). Secretion of VacA probably occurs via a mechanism analogous to that used for secretion of *Neisseria gonorrhoeae* IgA protease (14–15). Mature 87-kDa VacA monomers assemble into complex water-soluble oligomers typically comprised of 12 or 14 subunits (17–18). Upon exposure to acidic pH, these oligomers disassemble into monomeric components (17). Acidification of VacA enhances its cytotoxic activity and permits the toxin to insert into lipid membranes to form anion-conductive channels (19–23).

The mechanisms by which VacA causes alterations in cellular morphology and function are not yet well understood. Transfection of HeLa cells with plasmids expressing VacA results in cell vacuolation, which suggests that VacA has an intracellular site of action (24–27). Nearly all bacterial toxins that act intracellularly have an enzymatic activity, but thus far, no enzymatic activity of VacA has been identified. The formation of membrane channels by VacA also may contribute to cytotoxic effects, perhaps analogous to the mechanism by which aerolysin causes vacuolation of cells (28).

Structure-function analysis of VacA may be helpful in deci-

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TABLE I
H. pylori 60190-derived strains containing sacB/kan cassettes in vacA

H. pylori strain	Site of *sacB/kan* insertion in *vacA*[a]
VM064	*Bcl*I; 116
VM022	*Bsm*FI/*Eco*NI; 435–980
VM025	*Eco*NI/*Bst*XI; 980–1617
VM018	*Bst*XI/*Xcm*I; 1617–1901
VM028	*Xcm*I/*Bgl*II; 1901–2154
VM002	*Bgl*II/*Nhe*I; 2154–2551

[a] With the exception of strain VM064, *sacB/kan* cassettes replaced the *vacA* sequences originally present between the indicated restriction sites. Numbers designate nucleotides within the *vacA* open reading frame.

phering how this toxin exerts its cytotoxic effects. However, the construction of VacA mutants has been hindered by the inability to express a functional form of recombinant toxin in *Escherichia coli* (29). In this study, we utilized a recently developed mutagenesis method (30) to analyze the functional role of a unique strongly hydrophobic region near the VacA amino terminus. We report that a VacA mutant protein (VacA-(Δ6–27)) lacking this amino-terminal hydrophobic segment is indistinguishable from wild-type VacA in its secretion, assembly into oligomeric structures, and uptake by HeLa cells. However, this mutant protein is markedly altered in its capacity to form ion-conductive channels, lacks cytotoxic activity, and completely inhibits the vacuolating activity of the wild-type toxin.

MATERIALS AND METHODS

Bacterial Strains—*H. pylori* 60190 (ATCC 49503) was the parent strain used for construction of all mutants in this study. Characteristics of the *vacA* gene and the secreted VacA protein from this strain have been reported previously (13, 14, 17, 19). *H. pylori* strains were grown routinely on trypticase soy agar plates containing 5% sheep blood in room air containing 6% CO_2 at 37 °C.

Introduction of sacB/kan Cassettes into the vacA Gene of H. pylori 60190—The *sacB/kan* cassette from pKSF (30) was inserted into convenient restriction sites in plasmids containing *vacA* fragments from *H. pylori* 60190 (14). These plasmids were then used to transform *H. pylori* 60190 (14). Kanamycin-resistant transformants, in which the *sacB/kan* cassette had integrated into the *vacA* chromosomal locus via allelic exchange, were selected by growth on Brucella agar plates containing 5% fetal bovine serum and 30 μg/ml kanamycin. This approach resulted in the introduction of *sacB/kan* cassettes into six different sites within the chromosomal *vacA* gene of *H. pylori* strain 60190 (Table I).

Introduction of In-frame Deletion Mutations into the Chromosomal vacA Gene of H. pylori—Five different in-frame *vacA* deletion mutations were constructed by restriction endonuclease digestions of *vacA*-containing plasmids, followed by plasmid religations and transformation into *E. coli* DH5α. The restriction sites utilized were *Bsm*FI/*Eco*NI, *Eco*NI/*Bst*XI, *Bst*XI/*Xcm*I, *Xcm*I/*Bgl*II, and *Bgl*II/*Nhe*I (see Table I for locations within *vacA*). To maintain the open reading frames, a short oligonucleotide linker was inserted into two of these sites (*Bsm*FI/*Eco*NI and *Xcm*I/*Bgl*II). Six additional in-frame *vacA* deletion mutations were constructed by an inverse polymerase chain reaction approach. Briefly, oppositely oriented primers were chosen such that the 5′ nucleotides of each pair of primers defined the region to be deleted. Following thermal cycling, template plasmid DNA was eliminated by *Dpn*I digestion. Polymerase chain reaction products were end-polished with *Pfu* DNA polymerase, recircularized with T4 DNA ligase, and transformed into *E. coli* DH5α. Each plasmid was analyzed by nucleotide sequencing to verify that the desired deletion was present and that the open reading frames remained intact. Plasmids containing in-frame *vacA* deletions were used to transform the relevant *H. pylori* strains containing *sacB/kan*, and transformants were selected by growth on Colombia blood agar (Difco) plates containing 5% fetal bovine serum and 6% sucrose. Single colonies of sucrose-resistant, kanamycin-sensitive *H. pylori* were characterized by polymerase chain reaction size analysis to verify that the desired deletions were present. Fig. 1 depicts the procedure used for construction of one mutant strain, *H. pylori* AV452. A total of 11 different *H. pylori* strains, each containing in-frame deletions in *vacA*, were isolated.

Characterization of H. pylori vacA Mutants—Mutant *H. pylori* strains were grown in sulfite-free Brucella broth containing 0.5% charcoal, and proteins in the culture supernatants were concentrated by precipitation with a 50% saturated solution of ammonium sulfate (13). To determine whether mutant strains expressed and secreted VacA, both whole bacterial cells and concentrated broth culture supernatant proteins were immunoblotted with anti-VacA serum (13). Preparations of supernatant proteins also were tested in an antigen-detection ELISA[1] (13, 31), which permitted concentrated broth culture supernatant proteins from each mutant strain to be standardized according to VacA concentration. These standardized supernatant protein preparations were tested for vacuolating toxin activity in a HeLa cell assay, and cell vacuolation was assessed by light microscopy (13).

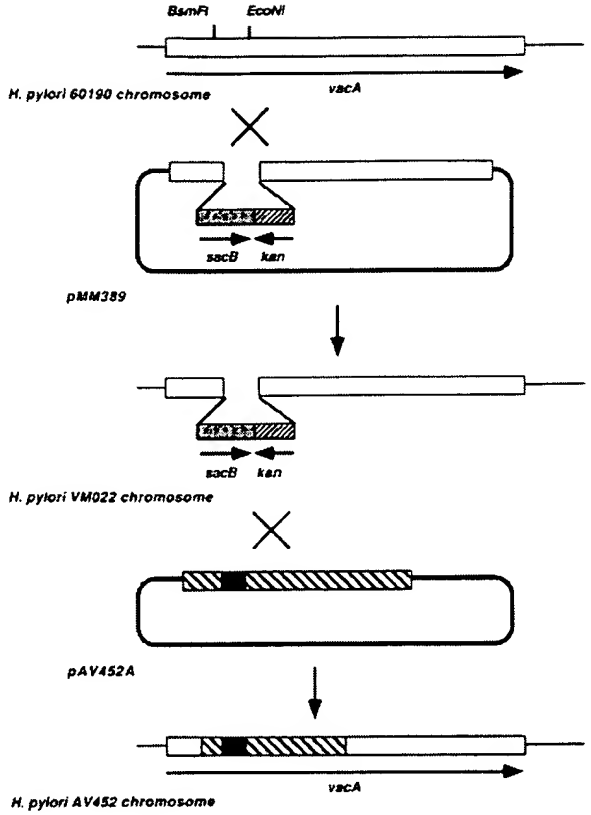

FIG. 1. **Construction of *H. pylori* AV452 using a *sacB*-based counter-selection approach.** The *sacB/kan* cassette from pKSF (30) was introduced into the *Bsm*FI and *Eco*NI sites of pA167, a plasmid containing a *vacA* fragment from *H. pylori* 60190, to yield pMM389. Transformation of *H. pylori* 60190 with pMM389 yielded a kanamycin-resistant colony designated *H. pylori* VM022. *H. pylori* VM022 was transformed with the plasmid pAV452A, in which nucleotides encoding amino acids 6–27 of VacA had been deleted (*dark box*). *H. pylori* transformants were selected by growth on 6% sucrose, and a strain (*H. pylori* AV452) containing the desired *vacA* deletion was isolated.

VacA Purification and Quantitation of Vacuolating Activity—VacA was purified from broth culture supernatants as described previously (17). Protein concentrations were determined using a Micro-BCA assay (Pierce). Unless otherwise stated, purified VacA preparations were routinely acid-activated before addition to HeLa cells. Acid activation was accomplished by dropwise addition of 250 mM HCl until a pH of 3 was reached (21). Purified, acid-activated VacA preparations were standardized by protein concentration and added to HeLa cells in minimal essential medium containing 10% fetal bovine serum and 10 mM ammonium chloride at 37 °C for 16 h. The vacuolating activity of purified VacA preparations was quantified using a neutral red uptake assay (32). Neutral red uptake data are presented as OD_{540} values (mean ± S.D.). Statistical significance was analyzed using Student's *t* test.

Atomic Force Microscopy—Purified VacA was added to supported lipid bilayers composed of a total lipid extract from bovine heart (Avanti Polar Lipids, Alabaster, AL), as described previously (19). The protein was injected into a buffer of 1 mM citric acid, pH 2.6, covering the

[1] The abbreviations used are: ELISA, enzyme-linked immunosorbent assay; DEPC, diethyl pyrocarbonate; GFP, green fluorescent protein.

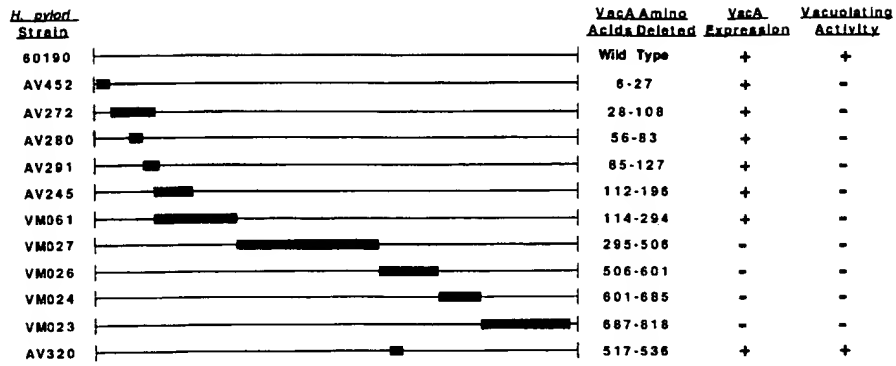

FIG. 2. **Construction and analysis of VacA deletion mutants.** Eleven different in-frame deletion mutations were introduced into the chromosomal *vacA* gene of *H. pylori* 60190. The *dark boxes* represent the corresponding regions that are deleted in each translated *vacA* product. The VacA amino acid numbering system is based on designation of the amino-terminal alanine residue of the mature toxin as amino acid 1. VacA expression was assessed by immunoblot analysis of culture supernatants and bacterial cells with anti-VacA serum. Vacuolating activity was assessed by testing concentrated supernatant proteins from each strain in a HeLa cell assay. The VacA content of VacA-containing supernatants was standardized based on results of an antigen-detection ELISA (13, 31).

supported bilayer. After incubating for 1 h, the sample was extensively washed and the pH was changed to ~7 to induce crystallization (19). The sample was briefly fixed with 2% glutaraldehyde, prior to imaging by atomic force microscopy (19). Imaging was performed in the contact mode with a Nanoscope II AFM (Digital Instruments, Santa Barbara, CA) using "twin tip" Si_3N_4 cantilevers. The typical scan rate was 7 Hz, and the applied force was minimized to 0.1 nN (19).

Electrophysiologic Analysis of VacA Channel-forming Activity—The planar lipid bilayers, composed of egg phosphatidylcholine/dioleoylphosphatidylserine/cholesterol (55:15:30 mol %) dissolved in *n*-decane, were prepared, and the membrane currents were measured as described previously (19, 20). The buffer in each experiment was buffer A (5 mM citric acid, pH 4.0, 2 mM EDTA), with the salt composition as described in the figure legends or tables. The potential is indicated relative to the cis-side, defined as the chamber to which the protein was added. Permeability ratios were determined from the Goldman-Hodgkin-Katz equation (33), after measuring the membrane voltage for zero current (reversal potential) in asymmetric salt concentrations. Statistical significance was analyzed using Student's *t* test.

Analysis of VacA Binding and Uptake by HeLa Cells—Purified VacA was iodinated using the IODO-GEN method (Pierce). IODO-GEN (2 μg) in chloroform was plated onto the wall of a microcentrifuge tube, and the chloroform was evaporated under a stream of N_2. To the IODO-GEN-containing tube, 1 mCi of [^{125}I]iodide in 50 mM sodium phosphate buffer, pH 7.2, and 50 μg of purified VacA were added in a final volume of 100 μl and incubated for 10 min at 25 °C. The liquid phase of the reaction was then removed, added to 10 mM non-radioactive iodide, and the free ^{125}I was removed by gel filtration on a 10-ml G-25 Sephadex column equilibrated with 10 mM Tris-buffered saline, pH 7.4, containing 1 mM EDTA and 25 μg per ml bovine serum albumin. This procedure resulted in effective radioiodination of VacA without a detectable loss of vacuolating activity.

HeLa cells were grown to confluency on 35-mm dishes. Acid-activated ^{125}I-VacA (500 ng/dish) was added to the cells for 3 h at 4 °C in Hepes-buffered saline (50 mM Hepes and 100 mM NaCl, pH 7.2) containing 1 mM $CaCl_2$, 1 mM $MgSO_4$, and 100 μg/ml bovine serum albumin. Cells then were washed three times to remove unbound VacA and were incubated for 4 h at 37 °C in Eagle's medium containing 10% fetal bovine serum and 10 mM ammonium chloride. In selected experiments, cells were treated with proteinase K (250 μg/ml for 30 min at 4 °C) to remove or digest surface-bound ^{125}I-VacA, and pelleted cells then were immediately lysed by boiling in SDS-polyacrylamide gel electrophoresis sample buffer. Proteins in cell lysates were separated by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography.

Inactivation of VacA by Treatment with DEPC—Diethyl pyrocarbonate (DEPC, Sigma) and purified VacA were mixed at pH 8.0 in a ratio of 100 DEPC molecules per VacA histidine residue (34). After incubation on ice for 1 h, the chemically modified VacA sample was mixed with an equal volume of minimal essential medium containing 10% fetal bovine serum. DEPC-treated samples were tested in HeLa cell assays within 1 h of preparation.

Transfection of HeLa Cells—HeLa cells were plated (200 μl) at a density of 5.0×10^4 cells per ml in 96-well tissue culture plates (Corning; Cambridge, MA) in Dulbecco's modified Eagle's medium supplemented with 2.5% fetal calf serum and 100 units penicillin/ml and 100 mg of streptomycin/ml. HeLa cells were first infected with recombinant vaccinia virus (vT7) bearing the T7 RNA polymerase gene (26). Vaccinia virus stock was trypsinized at 37 °C for 30 min and added to HeLa cells (26). After infection for 30 min, virus stock was removed, and the HeLa cells were transfected using the calcium phosphate method (26). Plasmids used for transfection included pET-20b containing an insert encoding residues 1–953 of VacA fused to GFP (26), pET-20b expressing the same VacA-GFP protein but with a 22-amino acid deletion (Δ6–27), or pET-20b encoding GFP only. Co-transfections were done by transfecting cells with a mixture of two different plasmid preparations in a 1:1 ratio. Mock-transfected cells were infected with vT7 and treated with transfection reagent only. Following the transfection procedure, the cells were incubated in Dulbecco's modified Eagle's medium plus 5 mM ammonium chloride at 37 °C for 20 h prior to analysis.

RESULTS

Expression of Mutant VacA Proteins—In an effort to construct an *H. pylori* VacA mutant protein that had altered functional properties but no gross alterations in structure, we introduced 11 different in-frame deletion mutations into the chromosomal *vacA* gene of *H. pylori* 60190, as described under "Materials and Methods." Each mutant strain was tested by immunoblot analysis (13) for the capacity to express and secrete VacA. Seven of these mutant strains expressed and secreted truncated *vacA* products of the expected size, but no *vacA* products were detected in either bacterial cells or supernatants from four mutant strains (Fig. 2). Concentrated culture supernatants from the seven VacA-expressing mutant strains were adjusted to a uniform VacA concentration based on the results of a VacA antigen-detection ELISA (13, 31), and these preparations then were tested for activity in a HeLa cell assay. Culture supernatant from *H. pylori* AV320 (containing VacA-(Δ517–536)) induced cell vacuolation, whereas each of the other mutant VacA proteins lacked detectable vacuolating activity. To determine whether these mutant VacA proteins could form water-soluble oligomeric structures, culture supernatant proteins from each mutant strain were fractionated by gel filtration chromatography, and high molecular mass fractions were immunoblotted with anti-VacA serum. VacA-(Δ6–27) and VacA-(Δ517–536) were detected in the same high molecular mass (>900 kDa) fractions in which wild-type VacA is typically found (13, 17), which indicated that these mutant proteins could form water-soluble oligomeric structures in a manner similar to wild-type VacA. In contrast, no high molecular mass oligomeric forms of the remaining 5 mutant VacA proteins were detected. Thus, only one mutant VacA protein (VacA-(Δ6–27)) was identified which seemed to be structurally intact, yet lacked vacuolating cytotoxic activity. This mutant VacA protein was selected for further detailed characterization.

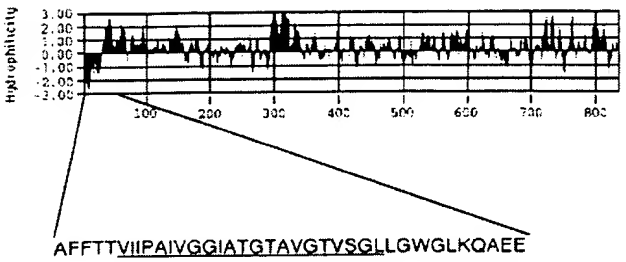

FIG. 3. **Hydrophilicity plot of the mature secreted VacA toxin from *H. pylori* 60190, generated by Kyte-Doolittle analysis.** The amino-terminal sequence of mature, secreted VacA is shown in *capital letters*. The *underlined* amino acids are located within a strongly hydrophobic region and were deleted in VacA-(Δ6–27).

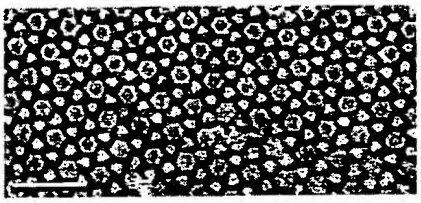

FIG. 4. **Structural analysis of VacA-(Δ6–27).** Purified VacA-(Δ6–27) was added to supported lipid bilayers composed of total lipid extract from bovine heart and incubated for 1 h at pH 2.6. After washing the membrane, the pH was raised to 7 to induce crystallization, and the crystals were analyzed by atomic force microscopy. The two-dimensional crystals produced by VacA-(Δ6–27) were indistinguishable from those of wild-type VacA (19). *Scale bar*, 50 nm.

Structural Characterization and Lipid Interactions of VacA-(Δ6–27)—The 22-amino acid deletion in VacA-(Δ6–27) is located within a unique region of strong predicted hydrophobicity near the amino terminus of VacA (Fig. 3). To examine structural properties of this mutant VacA protein, purified VacA-(Δ6–27) was incubated with supported lipid bilayers, and the bilayers then were imaged by atomic force microscopy (19, 35, 36). At pH values below 5, a high density of oligomeric mutant VacA associated with anionic lipid membranes (data not shown), in a manner similar to that observed for wild-type toxin (19). Adsorption of VacA-(Δ6–27) to the membrane at pH <5, followed by raising the pH to 7, resulted in the formation of two-dimensional crystal patches that could be imaged to a high degree of resolution (Fig. 4). All features of these crystals, including the lattice parameters, the inner diameter of the central rings, and the height by which the oligomers protrude from the bilayer, were identical to those described previously for wild-type VacA (19). These results indicate that the amino-terminal hydrophobic region of VacA is not required for oligomerization, association with lipids, or two-dimensional crystal formation and that the overall structure of the mutant toxin VacA-(Δ6–27) is not substantially different from that of wild-type toxin.

Electrophysiologic Properties of Channels Induced by VacA-(Δ6–27)—Wild-type VacA produces anion-conductive channels in lipid bilayers at low pH (19–20, 23). To determine whether VacA-(Δ6–27) was able to form similar channels, the purified mutant toxin was incubated with planar lipid bilayers at pH 4. Addition of the mutant toxin to lipid bilayers resulted in a macroscopic current that was detectable only after a much longer delay than when compared with the wild-type toxin under identical conditions ($p = 0.0047$) (Fig. 5). To determine whether the channel properties of the mutant differed from those of the wild-type toxin, the ion selectivities of the two types of channels were compared. The wild-type toxin channels were significantly more selective for anions than were mutant toxin channels ($p < 0.001$) (Table II). Single channel analyses

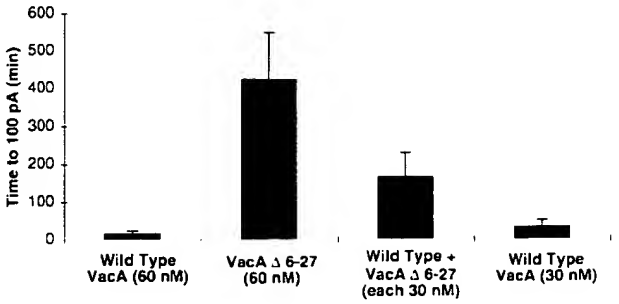

FIG. 5. **Kinetics of channel formation by wild-type VacA and VacA-(Δ6–27).** Mutant or wild-type VacA preparations (30 or 60 nM concentrations, as indicated) were added to planar lipid bilayers composed of egg phosphatidylcholine/dioleoylphosphatidylserine/ cholesterol (55:15:30 mol %) in buffer A with 100 mM NaCl. The time required to produce a current of 100 pA at −50 mV was then determined. In addition, results are shown for a 1:1 mixture of wild-type VacA and VacA-(Δ6–27) (each 30 nM). Results represent the mean ± S.D. from at least three independent determinations for each sample tested.

revealed that the conductance of mutant toxin channels was similar to that of the wild-type toxin channels (Table II). These results indicate that VacA-(Δ6–27) forms channels in lipid bilayers much less efficiently than wild-type VacA and that anion selectivity is diminished for mutant toxin channels. Therefore, the VacA amino-terminal hydrophobic domain is required for proper channel function.

Electrophysiologic Properties of Channels Formed by Mixtures of VacA-(Δ6–27) and Wild-type VacA—Acidification of wild-type VacA results in the disassembly of VacA oligomers into monomeric components (17, 22) and reassembly of monomers into oligomers can occur when VacA-containing solutions are shifted from acid to neutral pH (17). Therefore, we hypothesized that wild-type and mutant VacA monomers might assemble into hetero-oligomeric channels under the conditions of the planar lipid bilayer assay. To test this hypothesis, the two VacA species (each 30 nM) were mixed together at neutral pH, and the mixture then was acidified to pH 3 and maintained at this pH for 1 h before being added to planar lipid bilayers. The time required for the mixture to produce a current of 100 pA was significantly longer than that observed for wild-type VacA alone, regardless whether at 60 or 30 nM concentrations ($p < 0.01$), but was much shorter than that detected for 60 nM VacA-(Δ6–27) alone ($p = 0.005$). (Fig. 5). This latter observation indicates that within the period required for the mixture to generate 100 pA, few, if any, homo-oligomeric VacA-(Δ6–27) channels could form in the bilayer. Therefore, the macroscopic current produced by the mixture could arise via two possible mechanisms: (i) formation of hetero-oligomeric channels, or (ii) formation of primarily homo-oligomeric channels of wild-type VacA, with a delay caused by blockage of binding sites in the bilayer by the VacA-(Δ6–27) proteins. To discriminate between these alternatives, we determined the ion selectivity by measuring the reverse potential in asymmetric salt solutions. The channels formed by this mixture of VacA proteins exhibited a permeability ratio markedly different from that measured for homo-oligomeric channels of wild-type VacA ($p = 0.01$) (Table II). Taken together, these data suggest that the mixture of wild-type and mutant VacA proteins forms hetero-oligomeric channels.

Interactions of VacA-(Δ6–27) with HeLa Cells—To compare the cell-vacuolating activities of wild-type VacA and VacA-(Δ6–27), purified acid-activated proteins of each type were incubated with HeLa cells. Purified wild-type VacA caused the formation of large intracellular vacuoles, whereas purified VacA-(Δ6–27) lacked any detectable vacuolating activity for HeLa cells (Fig. 6). One possible explanation for the failure of

TABLE II
Comparison of VacA channel properties

VacA sample	P_{Na}/P_{Cl}[a]	Single channel conductance (pS)[b]
Wild-type VacA	0.19 ± 0.02 (n = 6)	159 ± 3 (n = 4)
VacA-(Δ6–27)	0.57 ± 0.05 (n = 8)	154 ± 31 (n = 4)
Wild-type VacA + VacA-(Δ6–27) (1:1)	0.38 ± 0.14 (n = 7)	ND[c]

[a] Macroscopic current was measured in buffer A with 100–150 mM NaCl using 30 nM wild-type VacA, 60 nM VacA-(Δ6–27), or a mixture of the two samples (each 30 nM). The number of experiments is designated (n). Results represent mean ± S.D.

[b] Current at −100 mV in buffer A with 1.5 M NaBr using 3 nM wild-type VacA or 45 nM VacA-(Δ6–27).

[c] ND, not determined.

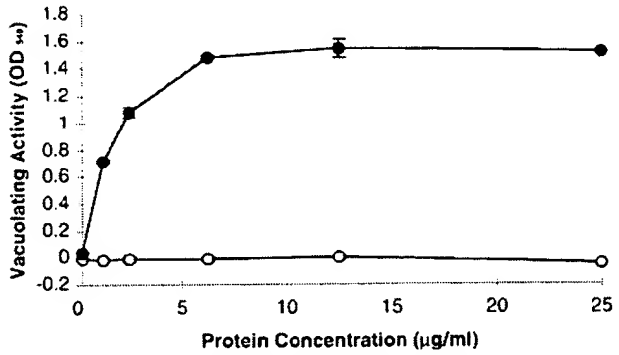

FIG. 6. **Vacuolating activity of wild-type VacA and VacA-(Δ6–27).** Purified acid-activated wild-type VacA (●) and acid-activated VacA-(Δ6–27) (○) were incubated with HeLa cells for 16 h at 37 °C. Vacuolating activity was quantified using a neutral red uptake assay (32). The wild-type toxin induced cell vacuolation, whereas the mutant VacA protein did not. Results represent the mean ± S.D. from triplicate determinations.

VacA-(Δ6–27) to induce cell vacuolation might be that HeLa cells fail to bind or internalize this mutant toxin. To test this hypothesis, we examined and compared interactions of purified radiolabeled wild-type VacA and VacA-(Δ6–27) with HeLa cells. Both forms of VacA bound to cells at 4 °C, and the surface-exposed ~87-kDa VacA proteins bound at this temperature were susceptible to digestion with proteinase K (Fig. 7, *lanes a* and *b*). After incubation of VacA proteins with cells at 37 °C for 4 h, both wild-type and mutant forms of the ~87-kDa toxin became resistant to proteinase K digestion (Fig. 7, *lanes c–f*). This inaccessibility to protease digestion provides strong evidence that both the wild-type and mutant forms of VacA are internalized by HeLa cells (27, 37). In the presence of a 100-fold excess of unlabeled wild-type VacA, there was a small reduction in the binding of both the radiolabeled wild-type and mutant VacA proteins to cells at 4 °C (data not shown). A high level of non-competable ("nonspecific") binding is perhaps attributable to VacA interactions with abundant cell-surface components, including anionic phospholipids (19, 23). A 100-fold excess of unlabeled wild-type VacA inhibited the cellular uptake of radiolabeled wild-type and radiolabeled mutant 87-kDa VacA bands to similar extents (Fig. 7, *lanes c–f*). Thus, compared with wild-type VacA, VacA-(Δ6–27) did not exhibit any detectable defects in binding or uptake by HeLa cells.

Inhibitory Effects of VacA-(Δ6–27)—We next investigated whether mixing VacA-(Δ6–27) with wild-type VacA resulted in alterations in the vacuolating cytotoxic activity of the wild-type toxin. Acid-activated wild-type VacA was mixed with varying concentrations of acid-activated VacA-(Δ6–27), and these preparations then were added to the neutral pH medium overlying HeLa cells. When the two proteins were present in equimolar concentrations, VacA-(Δ6–27) completely inhibited the activity

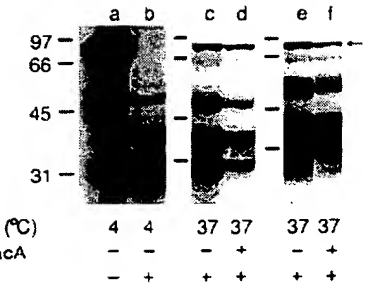

FIG. 7. **Binding and uptake of ^{125}I-VacA-(Δ6–27) by HeLa cells.** HeLa cells were incubated with purified acid-activated ^{125}I-VacA-(Δ6–27) for 3 h at 4 °C, and cells then were either treated with proteinase K (*lane b*) or left untreated (*lane a*). HeLa cells also were incubated with radiolabeled acid-activated wild-type VacA (*lanes c* and *d*) or radiolabeled acid-activated VacA-(Δ6–27) (*lanes e* and *f*) for 3 h at 4 °C in the presence or absence of a 100-fold excess of unlabeled acid-activated wild-type VacA, washed, incubated for an additional 4 h at 37 °C, and then treated with proteinase K. Cell-associated proteins were separated by SDS-polyacrylamide gel electrophoresis and analyzed by autoradiography. A protease-protected 87-kDa band (*arrow*) is visualized in *lanes c–f* but not *lane b*. Lower molecular mass radiolabeled bands represent proteolytic degradation products of VacA. The presence of a 100-fold excess of unlabeled acid-activated wild-type VacA during the 4 °C binding step inhibited the cellular uptake of radiolabeled wild-type and radiolabeled mutant 87-kDa VacA bands to similar extents (*lanes c–f*).

of wild-type toxin (Fig. 8). A significant inhibition also could be detected when the ratio of wild-type VacA to mutant toxin was 5:1 (Fig. 8). Acid-treated albumin was tested in similar concentrations as a control and failed to inhibit the vacuolating activity of wild-type VacA (data not shown). Treatment of VacA with DEPC yields an inactivated toxin that binds to cells but has markedly reduced cytotoxic activity (23). When equimolar concentrations of acid-activated DEPC-treated VacA and wild-type VacA were incubated with HeLa cells, no inhibition of wild-type VacA activity was detected (data not shown). Thus, VacA-(Δ6–27) exerted a dominant negative effect, whereas DEPC-treated VacA lacked this property.

Acid-activation of wild-type VacA results in markedly enhanced vacuolating toxic activity (17, 21). Therefore, we compared the capacity of acid-activated and untreated VacA-(Δ6–27) to inhibit wild-type VacA activity. Acid-activated VacA-(Δ6–27) completely inhibited the activity of the wild-type toxin, whereas non-acid activated VacA-(Δ6–27) had minimal inhibitory effects (Fig. 9). This suggests that the mutant toxin must undergo an acid-induced structural change before it can exert its dominant negative effect.

Intracellular Expression of VacA-(Δ6–27)—As shown in Fig. 7, VacA-(Δ6–27) did not exhibit any obvious defects in binding or entry into HeLa cells, which suggests that this mutant toxin is defective in intracellular activity. To test this hypothesis, HeLa cells that previously had been infected with vT7 were transfected with either pET20b harboring a gene encoding wild-type VacA fused to GFP (26) or pET20b harboring a gene encoding VacA-(Δ6–27)-GFP. Transfected cells were analyzed after 18 h for both vacuolating activity and GFP fluorescence (26). Fluorescence microscopy revealed that the wild-type and mutant proteins each were expressed within target cells. Intracellular expression of wild-type VacA resulted in cell vacuolation (detected by both light microscopy and neutral red uptake assay), whereas intracellular expression of VacA-(Δ6–27) produced no detectable morphologic changes (Fig. 10, $p < 0.001$).

We next tested the possibility that VacA-(Δ6–27) could inhibit the function of wild-type VacA when co-expressed within the same target cell. HeLa cells were co-transfected with plasmids encoding both VacA-(Δ6–27)-GFP and wild-type VacA, and the extent of vacuolation was determined by light micros-

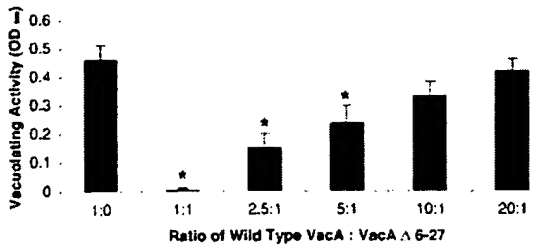

FIG. 8. **Inhibition of wild-type VacA cytotoxic activity by VacA-(Δ6–27).** Acid-activated wild-type VacA (5 μg/ml) was incubated with varying concentrations of acid-activated VacA-(Δ6–27) and then added to the medium overlying HeLa cells for 16 h at 37 °C. Vacuolating activity was quantified using a neutral red uptake assay (32). Results represent the mean ± S.D. from triplicate samples. *Asterisks* denote significant differences ($p < 0.05$) compared with wild-type VacA alone.

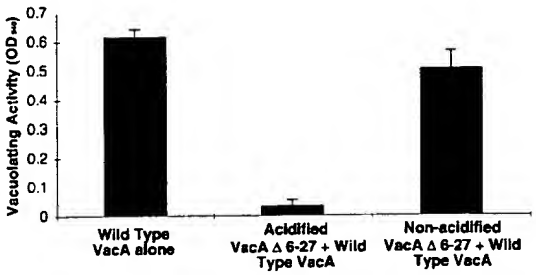

FIG. 9. **Acid activation of VacA-(Δ6–27) is required for inhibitory activity.** Identical aliquots of purified VacA-(Δ6–27) (5 μg/ml) were either acid-activated or left untreated and then added to tissue culture medium overlying HeLa cells. Acid-activated wild-type VacA (5 μg/ml) was added to the wells immediately thereafter. Vacuolating activity was quantified using a neutral red uptake assay (32). The acidified mutant VacA effectively inhibited wild-type VacA activity, whereas the non-acidified mutant failed to inhibit wild-type VacA activity. Results represent the mean ± S.D. from triplicate determinations.

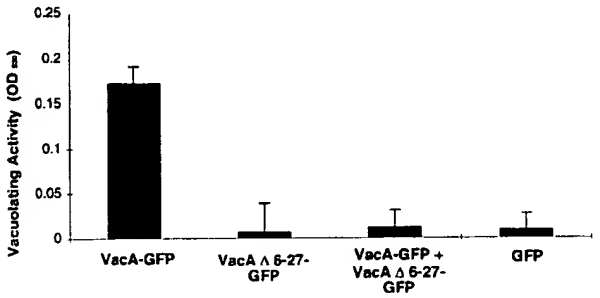

FIG. 10. **Intracellular expression of VacA-(Δ6–27).** HeLa cells were transfected with pET20b plasmids expressing VacA-GFP, VacA-(Δ6–27)-GFP, or GFP alone, as described under "Experimental Procedures." In addition, cells were co-transfected with plasmids encoding VacA-GFP and VacA-(Δ6–27)-GFP. After 20 h, the cells were assayed for uptake of neutral red (32). Background neutral red uptake detected with mock-transfected cells has been subtracted to yield net neutral red uptake. Results represent the mean ± S.D. from three separate experiments.

copy and by quantifying neutral red uptake. In these co-transfection experiments, a very low percentage (1–5%) of cells developed vacuoles, compared with 50–80% of cells transfected with plasmids encoding wild-type VacA alone. This difference was confirmed by neutral red uptake assay ($p < 0.001$) (Fig. 10). These co-transfection experiments indicate that VacA-(Δ6–27) can effectively block the vacuolating activity of wild-type VacA in an intracellular site.

DISCUSSION

The amino acid sequence of VacA and its effects on eukaryotic cells are unrelated to those of any other known bacterial protein toxin. Transfection of mammalian cells with plasmids encoding the amino-terminal 422 amino acids of VacA is sufficient to induce cell vacuolation (25, 26), and antibodies reactive with the carboxyl-terminal portion of mature secreted VacA (amino acids 509–836) inhibit VacA binding to cells (27). These results indicate that the amino-terminal portion of VacA corresponds to an intracellularly active domain, and the carboxyl-terminal portion may correspond to a cell-binding domain.

Additional efforts to analyze VacA structure-function relationships have involved the construction of VacA mutant proteins. VacA has not been expressed successfully as a functional recombinant protein in *E. coli* (29), and therefore, the construction and expression of VacA mutant proteins has been accomplished by manipulating the *vacA* gene in *H. pylori*. In previous studies, mutagenesis of two histidine residues (30) and mutagenesis of a surface-exposed domain corresponding to VacA amino acids ~327–372 (38) have failed to alter VacA activity. The only inactive VacA mutant constructed thus far has contained a large deletion (corresponding to amino acids 91–330) in the amino terminus of the toxin (39). This mutant VacA protein was secreted by *H. pylori* but formed dimers rather than typical six or seven-sided oligomers (39). Five of the mutant VacA proteins described in this study, each containing deletions in the region between amino acids 27 and 294, also failed to form typical oligomeric structures. The mechanistic basis for failure of these mutants to oligomerize properly is not clear, but we speculate that sequences located between amino acids 27 and 294 may directly mediate contact between adjacent monomers. Alternatively, it is possible that deletions in this region result in drastic alterations in VacA folding, thereby precluding proper oligomerization. Future studies involving site-directed mutagenesis may be helpful in clarifying whether residues 27–294 comprise an oligomerization domain. Notably, **all of the VacA mutants that fail to oligomerize properly lack vacuolating cytotoxic activity (Ref. 39 and this study), which** raises the possibility that VacA oligomerization may be essential for cytotoxic activity.

In contrast to the VacA deletion mutants discussed above, VacA-(Δ6–27) forms oligomeric structures similar to those of wild-type VacA. Moreover, VacA-(Δ6–27) binds to lipids and forms two-dimensional crystals with a structure indistinguishable from that of wild-type VacA. Collectively, these data indicate that the overall folding of VacA-(Δ6–27) remains intact despite the presence of a 22-amino acid deletion. VacA-(Δ6–27) does not exhibit any obvious defects in binding or uptake by HeLa cells but fails to induce vacuole formation. The inability of VacA-(Δ6–27) to induce vacuolation when expressed intracellularly suggests that this mutant toxin is primarily defective in intracellular function.

Several previous studies have proposed that formation of intracellular membrane channels is important for the morphogenesis of VacA-induced cell vacuoles (19, 20, 23). The loss of vacuolating activity and alteration of channel-forming activity that both result from deleting the VacA amino-terminal hydrophobic segment provide evidence in support of this hypothesis. One unresolved issue related to understanding the role of channel formation in VacA cellular intoxication concerns the role of acidic pH in VacA activation. Specifically, VacA channel formation in planar lipid bilayers requires exposure of the toxin to acidic pH (19, 20, 23). In contrast, VacA expressed intracellularly in mammalian cells effectively forms vacuoles without any apparent exposure of the protein to acidic pH. This apparent discrepancy could be related in part to the fact that VacA is purified from *H. pylori* supernatants in an oligomeric form (13, 17, 18), whereas VacA may exist predominantly in a monomeric form when expressed within mammalian cells. We spec-

ulate that the formation of VacA membrane channels may involve oligomerization of membrane-bound monomers (43), and therefore, the role of acidic pH in VacA channel formation *in vitro* may simply be to disrupt VacA oligomers into monomeric components (17).

A remarkable property of VacA-(Δ6–27) is its capacity to inhibit the cytotoxic activity of the wild-type toxin. One possible explanation for this phenomenon is that VacA-(Δ6–27) might competitively inhibit the binding of wild-type toxin to a putative VacA receptor on the surface of cells (40–42). However, inhibition of wild-type toxin activity was detectable when the ratio of wild-type toxin to mutant VacA was 5:1. Typically, a substantial excess of mutant protein is required to inhibit binding of an active ligand to cell-surface receptors. Moreover, the strongest evidence against competitive inhibition at a cell-surface site is that intracellular expression of a VacA protein containing the Δ6–27 deletion inhibited the vacuolating activity of wild-type VacA.

A more likely explanation for the dominant negative phenotype exerted by VacA-(Δ6–27) is the formation of dysfunctional mixed oligomers, comprised of both mutant and wild-type VacA monomeric components. This model is consistent with the capacity of purified mutant VacA to inhibit wild-type VacA activity when the ratio of wild-type to mutant VacA is 5:1 (*i.e.* a dominant negative effect). Indeed, the capacity of VacA-(Δ6–27) to alter dramatically the channel-forming activity of wild-type VacA provides evidence that these two species can interact to form dysfunctional hetero-oligomeric structures. Further insight comes from the observation that VacA-(Δ6–27) can exert a dominant negative phenotype when co-expressed with wild-type VacA from within target cells. This raises the possibility that an intracellular oligomeric form of VacA might be required for vacuolating cytotoxic activity. Intracellular interactions between VacA molecules potentially could be critical for the formation of membrane channels or for establishing a quaternary structure with unique binding or enzymatic properties. However, at this time we cannot rule out alternate interpretations of the data. For example, VacA-(Δ6–27) might form oligomers more readily than wild-type VacA, and formation of intracellular hetero-oligomers might deplete the intracellular pool of active monomeric wild-type VacA. Alternatively, compared with wild-type VacA, VacA-(Δ6–27) might exhibit increased avidity for an intracellular target molecule. Further construction and analysis of VacA mutants should be helpful in clarifying the intracellular mechanisms by which VacA alters cellular function.

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A *Helicobacter pylori* Vacuolating Toxin Mutant (VacA Δ49-57) Which Fails to Form Oligomeric Structures Presents a Dominant Negative Phenotype

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Abstract.

Most *Helicobacter pylori* strains secrete a toxin (VacA), which is one of the major virulence factors thought to play an important role in the pathogenesis of *H. pylori*-associated gastroduodenal diseases. Previous reports have shown that the VacA amino terminal region is important for the formation of large cytoplasmic vacuoles, the most prominent effect of the toxin. The purpose of this study was to further understand the role of N-terminal regions protected from proteolysis when VacA interacts with artificial membranes. Using a counterselection system, we constructed a *H. pylori* strain SPM 326-Δ49-57, which produces a mutated toxin containing a deletion of 8 amino acids in one of these protected regions. VacA Δ49-57 was correctly secreted by *H. pylori* but failed to oligomerize and did not present any detectable vacuolating cytotoxic activity. However, the mutated toxin was internalized and stained the perinuclear region of HeLa cells. Moreover, when an equimolar mixture of purified VacA Δ49-57 and wild-type VacA were added simultaneously to HeLa cells, the mutant toxin exhibited a dominant negative effect, completely inhibiting the vacuolating activity of wild-type VacA. This dominant negative effect was observed only with non-acid activated VacA Δ49-57 whereas acid activated mutated molecules had minimal inhibitory effects. This loss of activity was correlated with the disappearance of oligomers in electron microscopy.

We propose a model in which VacA Δ49-57 forms unstable heterodimers with wild type toxin and blocks the reformation of VacA oligomers after acid exposure, resulting in the loss of VacA cytotoxicity.

Introduction.

Helicobacter pylori is a Gram-negative, microaerophilic, spiral-shaped bacterium that colonizes the gastric mucosa of half world's population (1). This bacterium is associated with gastritis and peptic ulcers (2) and for long-term chronic infection, with the development of gastric carcinoma and MALT[1] (3).

Most *H. pylori* strains produce and secrete a toxin (VacA) which together with two other virulence factors, CagA (4) and BabA (5) is significantly associated with strains isolated from patients with more severe disease (6). The most extensively characterized activity of VacA is its capacity to induced vacuolation in mammalian cells (7). These vacuoles contain markers for late endosomes and lysosomes and have an acidic intracellular pH (8,9). Vacuolation is not the only function of VacA. When added to polarized epithelial cell monolayer, it induces an increase in monolayer permeability for various ions and small uncharged molecules (10). After internalization by cells, it can provoke cytochrome c release into the cytosol, which leads to apoptosis (11-14). VacA also interferes with the immune system, by altering the process of antigen presentation (15) and recently, it has been shown to inhibit T cell proliferation by two different independent mechanisms (16-18). Moreover, when administered intragastrically to mice, it causes gastric epithelial erosion (19,20).

The *vacA* gene produces a protoxin of 140 kDa which is processed during export to the surface to yield a mature secreted toxin of 87 kDa (7,19). A molecule of VacA has two domains, p37 and p58, connected by a hydrophilic loop that can be cleaved after release from the bacterium. After cleavage the two fragments can remain non covalently

associated (19). The p58 domain is responsible for host cell binding (21) and host tropism (22), whereas the p37 domain, together with the N-terminal 192 amino acids of p58 is sufficient to cause vacuolation when expressed in the cytoplasm of host cells (23). Mature 87 kDa VacA monomers assemble into water-soluble high molecular weight oligomer containing 6-7 or 12-14 copies of the mature toxin polypeptide (24-26). The purified oligomeric form of the toxin is biologically inactive, but after exposure to acidic pH, it disassembles into monomeric components (25) which permits insertion of the toxin into lipid membranes to form anion-conductive channels (27,28). The VacA channel formation induces an osmotic imbalance of intracellular acidic compartments, leading to cytoplasmic vacuolation.

A previous study has reported that a mutated toxin of VacA lacking the hydrophobic amino terminus region presents a dominant negative phenotype (29). This mutant, which lacks cytotoxic activity and completely inhibits the vacuolating activity of the wild type toxin, is indistinguishable from wild type VacA in its secretion, assembly into oligomeric structures and uptake by HeLa cells. It was demonstrated later that this mutant lacks three tandem GXXXG motifs necessary for dimerization of VacA within membranes and consequent anion channel formation (30).

In this study, we describe another dominant negative mutant, VacA Δ49-57, which has an 8 amino acids deletion in a region known to be protected from proteolysis due to its interaction with artificial membranes. This *in vitro* assay system is used to mimick VacA interaction with host membranes (31). This mutant presents the same inhibition of the activity of wild type VacA, however it fails to form oligomeric structures. Taken

together, our data demonstrate that the mutated toxin has the capacity to block the reformation of the oligomeric structure of native VacA after exposure to acidic pH, and consequently, blocks the vacuolating activity.

Materials and Methods.

Bacterial and Yeast strains, culture condition

H. pylori CCUG 17874 was used as the source of VacA. *H. pylori* SPM 326 that encodes a s1m1 type *vacA* (32,33) was the parental strain used for construction of the mutant. Colonies of *H. pylori* grown on blood agar plates (Columbia agar with 5% horse blood) were inoculated into Brucella broth containing 0.2% β-cyclodextrin and were cultured for 2 days at 170 rpm in microaerophilic conditions. Yeast two-hybrid experiments were performed with the *Saccharomyces cerevisiae* EGY48 (*MATα ura3 trp1 his3* 6*LexA-operator-LEU2*) strain transformed with the plasmid pSH18-34 containing the *lacZ* reporter gene. Yeast strains were grown in synthetic minimal medium (SD), supplemented with the required amino acids at 30°C as described (34).

Construction of H. pylori SPM 326-Δ49-57

First, a recipient strain was created by introduction of the *kan-sacB* cassette into the *vacA* gene of *H. pylori* SPM 326. The *kan-sacB* module amplified from the plasmid pEnKSF (35) was inserted into the *Nco*I/*Eco*NI site of pBlueScriptKS p37, a plasmid containing a substantial portion of the *vacA* SPM 326 gene to create pKan/sacB (Fig. 1). The *Nco*I site, which is absent in the *vacA* gene, was added, using the QuickChange Site-Directed Mutagenesis Kit (Stratagene) by changing two nucleotides just before the sequence coding the translational start site. A kanamycin-resistant, sucrose-sensitive clone, in which the expression of the VacA molecule was inactivated, was selected. This strain, *H.*

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pylori SPM 326KO2, was then transformed by pBlueScriptKS p37-Δ49-57 and a sucrose-resistant, kanamycin-sensitive, transformant coming from homologous recombination between the *vacA* fragment was selected as summarized in figure 1 (35). The strain was analyzed by nucleotide sequencing to verify that the desired mutation was present.

The plasmid pBlueScriptKS p37-Δ49-57 was obtained from pBlueScriptKS p37 by introduction of an in frame *vacA* deletion mutations using the QuickChange site directed mutagenesis kit (Stratagene). Briefly, oligonucleotides primers each complementary to opposite strands of the vector and containing the deletion were extended by polymerase chain reaction using *Pfu* DNA polymerase. Following thermal cycling, template DNA was eliminated by *Dpn*I digestion and the PCR products were used to transforme *E. coli* XL1-Blue.

Purification of VacA and VacA Δ49-57

VacA from *H. pylori* strain CCUG 17874 was purified from the broth culture supernatant as described (36). For VacA Δ49-57, the biomass from 1 liter culture of *H. pylori* SPM 326-Δ49-57 was removed by centrifugation at 11,000 x *g* for 20 min. The supernatant was passed through a 0.2 μm filter and then applied at a flow rate of 1 ml/min to a column (5 by 3.5 cm, XK column; Amersham Pharmacia Biotech) containing Matrex Cellufine Sulfate (Amicon). The proteins were washed extensively with 100 mM NaCl-20 mM phosphate buffer (pH 6.5). VacA Δ49-57 was eluted from the column with 350 mM NaCl-20 mM phosphate buffer (pH 6.5). The eluate was brought to 50% saturation

with ammonium sulfate and centrifuged at 20,000 x *g* for 30 min. The pelleted proteins were resuspended in PBS and dialyzed extensively against the same buffer. Purified toxins were stored at 4°C and the concentrations were determined using a Micro-BCA assay (Pierce).

Cell vacuolation assay

HeLa cells were seeded at 1.5 x 10^5 ml^{-1} in 96-well plates in DMEM containing 10% FCS and 2 mM glutamine at 37°C in 5% CO_2 16 h before the assay. Before addition to cells, purified toxin preparations were acid-activated by adjusting to pH 2.0 for 5 min at room temperature and then neutralized as described previously (37). The extent of vacuolation was determined quantitatively by measuring the uptake of neutral red dye after incubation of the toxin with cells at 37°C for 9 h in DMEM containing 2% fetal calf serum and 15 mM ammonium chloride (7).

Deep etch electron microscopy

VacA and VacA Δ49-57 molecules were prepared for microscopy by a procedure of absorption to mica followed by quick-freeze deep etching (38). The samples were processed as described (24). For the experiment showing the effect of VacA Δ49-57 on VacA, an equimolar concentration of each toxin, acid activated when indicated, were mixed before the treatment.

Glycerol gradient centrifugation analysis

For analysis, 14 ml 10-30% glycerol gradients were prepared in either 60 mM Tris pH 7.5, containing 100 mM NaCl, or 100 mM glycine, pH 3.0, containing 100 mM NaCl. Samples of 200 µl (dialyzed ammonium sulfate-precipitated proteins from broth culture supernatant diluted to OD_{600} = 1) were layered on the gradients and centrifuged at 39,000 rpm for 10 h at 4°C in a an SW40 Ti rotor (Beckman Instruments) as described previously (25). For the experiment showing the effect of VacA Δ49-57 on VacA, 100 µl of each concentrated supernatant, acid activated when indicated, were mixed before centrifugation. For the control, 100 µl of SPM 326 acid activated concentrated supernatant were mixed with 100 µl of SPM 326KO2. Gradients were fractionated from the top using a Piston Gradient Fractionator (Biocomp). Proteins from the fractions were precipitated with 10 % TCA and 0.02 % sodium deoxycholate. The presence of VacA and VacA Δ49-57 was detected by Western blotting with rabbit anti-VacA serum (19).

Immunofluorescence and confocal microscopy

For indirect immunofluorescence analysis, HeLa cells were grown on chamber slides (Nunc). After 4 h incubation with 5 µg/ml of purified VacA Δ49-57 the cells were fixed with 3.7% paraformaldehyde in PBS plus 5 mM Ca^{2+} and Mg^{2+} for 15 min. Aldehyde groups were quenched with 0.2 M glycine for 5 min. For cytosolic staining, cells were permeabilized with 0.2% Triton X-100 for 10 min at room temperature (39). PBS containing 5% FCS was used as blocking solution. For VacA Δ49-57 staining, a mouse

monoclonal antibody against the native toxin was used at 2 μg/ml (40). Alexa 488 goat anti-mouse (Molecular Probes) was used as secondary antibody. Slides were mounted with *SlowFade Light* Antifade kit (Molecular Probes). Confocal images were obtained using a Leica TCS4D confocal microscope equipped with a krypton/argon laser (Leica Microsystems).

Cross-linking experiments

A fixed volume (20 μl) of acid activated SPM 326 concentrated supernatant was incubated with 0, 60 or 100 μl of SPM 326-Δ49-57. Samples were diluted using SPM 326KO2 concentrated supernatant to a final volume of 120 μl when necessary and incubated 30 min at room temperature. Each preparation was then treated by adding a solution of PBS, 25 mM lysine 1.6 mM formaldehyde or PBS, 25 mM lysine for 48 h at 37°C, followed by dialysis against PBS (41). Samples were analysed by immunoblotting using a polyclonal rabbit antiserum after separation on a 3-8% SDS PAGE.

Yeast two hybrid system

Sequences encoding the wild-type p37, p37 Δ49-57 and p58 VacA domains were PCR amplified from pBlueScriptKS p37, pBlueScriptKS p37-Δ49-57 and pBlueScriptKS p58 (Skibinski et al.) respectively. Products were cloned into plasmids encoding the transcription activation domain B42 (pJG4-5L) and/or the LexA DNA binding domain (pEG202-NLS) (34,42). Primers were designed in order to amplify amino acids 1 to 319

(p37 domain) and 320 to 837 (p58 domain) of the mature toxin from *H. pylori* SPM 326. Before transformation, the plasmids were analyzed by automated nucleotide sequencing. The EGY48/pSH18-34 yeast strain was co-transformed with 2 μg of bait and prey containing plasmids in all possible combinaitions using the lithium acetate method (43) and cultured at 30°C on SD -Trp, -His, +Leu. In order to test whether interactions between VacA domains could be detected in this system, and which combination was able to activate transcription of the *LEU2* and the *lacZ* reporter genes, several independent clones of each co-transformant, were seeded on selective plate. Three isolates of each co-transformant were then chosen for the β-Galactosidase interaction assay.

To quantify the level of interaction between the different domains, selected co-transformants were grown in 4 ml SD medium containing 2% galactose 1% raffinose and 50 μg/ml leucine at 30°C for 24 h until the culture reached $OD_{640} = 4$. For each strain a volume of culture containing 10^7 cells, corresponding to an OD_{640} of 2, was centrifuged at 3000 x *g*, 5 min, washed once with 1 ml of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1.8 mM $MgSO_4$, 0.27% β-Mercaptoethanol, pH 7) (44) and the pellet was resupended in 150 μl of Z buffer. 50 μl aliquots of this cell suspension were permeabilized using Z buffer containing 70% $CHCl_3$, 0.02 % SDS and incubated with 700 μl of ONPG (2 mg/ml) at 30°C. The reaction was stopped at time intervals by the addition of 500 μl of 1M K_2CO_3 and the total reaction time was recorded. The OD_{420} values of clarified reaction supernatants were measured. The β-galactosidase activity was

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calculated using the Miller equation (34). The values presented are the average of three independent co-transformants that were assessed in three different experiments.

Results.

VacA Δ49-57 is devoid of vacuolating activity

In order to characterize a p37 region known to interact with artificial membranes (31) and its possible implication in the internalization of the toxin, we used a counterselection marker (35,45) to introduce an 8 amino acids in frame deletion into the chromosomal *vacA* gene of *H. pylori* SPM326 (Fig.1). Western blot analysis of soluble extracts and supernatants showed that the mutated strain *H. pylori* SPM 326-Δ49-57 produces and secretes a toxin indistinguishable from the wild type VacA (data not shown). However, purification of VacA Δ49-57 was obtained using a different procedure (see Materials and Methods). VacA Δ49-57 was eluted from the Matrex Cellufine Sulfate column with 350 mM NaCl, whereas wild type VacA needs a higher salt concentration (36). This difference of affinity for the Matrex Cellufine Sulfate resin implies that the mutated toxin presents different properties compared to VacA. This allowed us to purify VacA Δ49-57 in one step.

The amino acids deleted are located in the p37 domain, known to be essential for the vacuolating activity (Fig. 2). Purified VacA Δ49-57 was tested for activity in a neutral red uptake assay using HeLa cells. As shown in figure 3, the mutated toxin did not induce formation of vacuoles.

VacA Δ49-57 enters epithelial cells

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VacA is capable of entering the cell cytosol where it displays its activity (46). We investigated if the lack of vacuolation observed was due to the incapacity of VacA Δ49-57 to be internalized by HeLa cells. After 4 h infection, cells treated by the toxin showed labeled spots in the perinuclear region (Fig. 4). The staining occurs only under permeabilized condition, demonstrating the capacity of VacA Δ49-57 to be incorporated by epithelial cells like wild type VacA.

VacA Δ49-57 fails to form the oligomeric structure

Purified VacA forms water-soluble oligomeric structures which can be observed by transmission electron microscope after quick freeze, deep etch preparation (24). Since VacA Δ49-57 has a lower affinity for the Matrex Cellufine Sulfate resin we checked the integrity of the mutated toxin. Transmission electron microscope visualization of purified VacA Δ49-57 did not show any structure with either six or seven fold radial symmetry observed with the native cytotoxin (Fig. 5). Under acidic treatment, VacA oligomers disassemble into monomers and the two structures can be separated by velocity sedimentation on glycerol density gradients (25). As expected the pattern of sedimentation of SPM 326 concentrated supernatant was different between acidic and neutral pH whereas it was exactly the same for SPM 326-Δ49-57 (Fig. 5). This demonstrates that VacA Δ49-57 fails to form oligomeric structures at neutral pH and that the structure observed by transmission electron microscope is the monomer.

VacA Δ49-57 is a dominant negative mutant

The vacuolating activity of the native toxin can be abolished by introducing mutated VacA into the oligomer (29). We next investigated if mixing wild type VacA with VacA Δ49-57 can interfere with the formation of vacuoles. A fixed concentration of acid activated VacA was incubated with increasing concentrations of VacA Δ49-57, acid activated when indicated, before addition to the medium overlying HeLa cells. VacA Δ49-57 clearly affects the cytotoxic activity of wild type VacA in a dose dependant manner (Fig 6). When the two toxins were present in equimolar concentrations, VacA Δ49-57 completely inhibited the activity of the native protein. Heat inactivated VacA Δ49-57 (10 min at 95°C) was also tested in an equimolar concentration but did not present any inhibitory effect (data not shown). Surprisingly, the pattern of inhibition of VacA Δ49-57 was dependent on acid treatment (Fig. 6). Non acid activated VacA Δ49-57 completely inhibited the vacuolating activity of wild type VacA whereas acid activated VacA Δ49-57 affected the cytotoxic activity only by 50 %. Thus VacA Δ49-57 exerted a dominant negative effect only when it was not acid activated.

VacA Δ49-57 disturbs wild type VacA oligomer reformation

In order to explain the dominant negative phenotype of VacA Δ49-57, we next sought to determine whether VacA monomers produced by acidification could reassemble into oligomers in the presence of the mutated toxin. A solution with equimolar concentrations of VacA Δ49-57 and VacA was visualized by transmission electron microscope after

quick freeze, deep etch preparation. The electron micrograph presented two kinds of structure, the oligomer characteristic of native VacA and a smaller structure also observed in figure 5 corresponding to the VacA Δ49-57 monomer (Fig. 7A). When VacA was acidified 5 min at room temperature and neutralized before addition of mutated toxin, the high molecular weight structure disappeared (Fig. 7B). In order to prove that the absence of oligomers was due to VacA Δ49-57 and not to acid treatment, purified acidified VacA was also submitted to quick freeze, deep etch preparation. As shown in figure 7C, this procedure yielded a relatively disorganized flower-shaped structure similar to the VacA oligomers already described (25). Moreover, glycerol gradient sedimentation experiments on concentrated culture supernatant (Fig. 7C lower panel) demonstrated that VacA Δ49-57 was responsible of the phenomenon since concentrated supernatant of SPM 326-Δ49-57 induced monomerisation of VacA in SPM 326 acid activated preparations whereas SPM 326KO2 did not (Fig. 7C lower panel). In conclusion, loss of vacuolating cytotoxic activity of wild type VacA in the presence of an equimolar amout of VacA Δ49-57 was correlated with the disappearance of high molecular weight structures in solution, and this inhibitory effect was dependent on the acid activation of native VacA.

Low concentration of formaldehyde in the presence of free lysine has been succesfully used to cross-link VacA monomers and detoxified the toxin (41). To further understand the process by which VacA Δ49-57 blocks VacA oligomer reformation, we incubated SPM 326 acid activated concentrated supernatant with equal amounts or an excess of

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SPM 326-Δ49-57 supernatant before treatment with formaldehyde in the presence of free lysine. As shown in figure 8, formaldehyde treatment of wild type toxin resulted in the appearance of high molecular mass species corresponding to the expected size of the dimer and the trimer, and disappearance of the band corresponding to the VacA monomer. In the presence of VacA Δ49-57, the amount of mononer increased since the mutated toxin does not oligomerize. Surprisingly, the dimer appears to be prevalent whereas the trimer disappeared. This effect was accentuated when the mutated toxin was in excess.

Interaction of p37/p58 and p37Δ49-57/p58 in a yeast two-hybrid system.

Recently, it has been shown that the yeast two-hybrid system can be used to characterize interactions between the two domains of VacA, p37 and p58 (47). Since the Δ49-57 deletion in the p37 domain affects the interaction between the VacA domains necessary for the formation of oligomers, we decided to use this system to test if the interactions between the p37 and p58 domain were compromised by the deletion. *vacA* sequences encoding p37 and p58 fragments were cloned into plasmids containing the DNA binding domain of LexA (pEG202-NLS) and/or the transcription activation domain B42 (pJG4-5L) (34). EGY48/pSH18-34 yeast strain was transformed with all possible combinations of the p37 and p58 plasmids. Co-transformants were plated on specific medium for positive selection of protein-protein interactions. All co-transformants containing a p58 hybrid were able to grow on medium lacking leucine even in the absence of the B42 activating domain (data not shown). This suggests that, the p58 fragment by itself could

activate transcription. However, when yeasts were transformed with p37 cloned in pEG202-NLS, the reporter genes were activated only in the presence of the plasmid pJG4-5L-p58. This combination was used as a positive control for further investigation of p37 Δ49-57/p58 interactions.

A β-galactosidase liquid assay using yeast extracts was performed to characterize the p37 Δ49-57/p58 interactions (Fig. 9). The β-galactosidase activity obtained with yeast extracts of stains co-transformed with plasmids pEG202-NLS-p37 Δ49-57 and pJG4-5L-p58 was lower than the activity of yeast expressing wild type p37. Thus, the 8 amino-acids deletion in the p37 domain showed a 50 % decrease in the interaction with p58. Yeast co-transformed with pEG202-NLS-p37/ pJG4-5L or pEG202-NLS-p37 Δ49-57/ pJG4-5L did not present any β-galactosidase activity whereas the strain transformed with pEG202-NLS and pJG4-5L-p58 showed a low activity. This confirms the previous observation that the p58 fragment gives a weak activation of transcription in this experimental system.

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Discussion.

Use of a counterselectable *sacB* gene from *Bacillus subtilis* has been successful in the past for construction of *H.pylori* strains producing mutated VacA cytotoxin (21,29,35,47). Here, we used the same system to study a region known to be protected from proteolysis because of its interaction with artificial membranes (31).

Purified VacA assembles in high molecular weight oligomers which have a flower-like shaped structure when observed by deep etch electron microscopy (24-26). Mutational analysis of p37 has shown that the region spanning amino acids 28-196 is important for interaction between two adjacent monomers and formation of the global structure (29,47). In this study, we show that the region known to be protected from proteolysis, (amino acids 49 to 57) is involved in the p37/p58 interaction of two different monomers.

The mutated VacA protein described in this study was produced and secreted by *H. pylori* in a manner similar to the wild type toxin but lacked vacuolating cytotoxic activity. This absence of activity corralated to the incapacity of the toxin to oligomerize, which confirms that VacA oligomerization is essential for vacuole formation as already described (21,29). So far, this is the smallest deletion capable of blocking the oligomerization of VacA. We cannot exclude that this deletion may perturb the correct folding of the protein. But it is known that p58 expressed independently of p37 is exported correctly and interacts with the surface of target cells (21), suggesting an independent folding of the two domains . So we suppose that this deletion can only perturb p37 global structure.

A previous study showed that the p58 molecule was able to interact with the surface of target cells but was not internalized (21). This suggests that either the p37 subunit or the ability to form an oligomeric structure is required for cell entry. Here we describe that the VacA Δ49-57 monomer has the capacity to enter HeLa cells and localise in the cytoplasm in a manner similar to what is observed in VacA infected AGS cells (39), demonstrating that oligomerization is not necessary for internalization. This mechanism differs from that described for the C2 toxin of *Clostridium botulinum*, a related channel forming molecule which needs to form oligomers for cellular uptake (48). We propose that a region of p37, different from the region deleted in this study, and still unknown must be involved in VacA internalization.

A remarkable property of VacA Δ49-57 is its capacity to inhibit the cytotoxic activity of the wild type toxin. This phenomenon was probably not due to competition for the binding to the VacA receptor. It is known that the p58 domain is responsible for binding to the host cell and can fold independently of p37 (21), so VacA Δ49-57 is expected to bind the VacA receptor with the same affinity as the wild type toxin. When the two proteins were mixed in equimolar concentrations, a 50 % inhibition of cytotoxic activity would be expected. In our case the inhibition was 100 %. Moreover, HeLa cells exhibit a high level of nonspecific binding for radiolabeled VacA. Only a small reduction in the activity was observed in the presence of a 100-fold excess of unlabeled VacA (49,50). VacA Δ49-57 was able to abolish the cytotoxic activity of wild type VacA in a manner similar to VacA Δ6-27, another dominant negative mutant of *H. pylori* vacuolating toxin

(29). VacA Δ6-27, however, presented a dominant negative phenotype only when it was acid activated, while VacA Δ49-57 was able to inhibit only 50% of VacA activity under those conditions. VacA Δ6-27 forms an oligomer, and after exposure to low pH, monomers of VacA Δ6-27 can interact with monomers of VacA to form dysfunctional mixed oligomers (29). In contrast, VacA Δ49-57 does not oligomerize and does not need to be acid activated to interact with wild type VacA monomers. We suppose that the difference of inhibition observed between acid activated and non acid activated VacA Δ49-57 was the result of a structural damage of the mutated toxin after exposure to acidic pH.

Loss of cytotoxic activity of wild type VacA was correlated with disappearance of VacA oligomers as shown by quick freeze, deep etching electron microscopy, and appearance of a major band corresponding to the molecular mass of a VacA dimer in SDS PAGE after formaldehyde treatment. We propose a model that could explain the dominant negative phenotype of VacA Δ49-57 (Fig. 10). In the presence of equimolar concentrations of VacA and VacA Δ49-57, the p37 domain of the native toxin can bind the p58 domain of the mutated toxin, leading to the formation of a heterodimer. Moreover due to the deletion in p37, VacA Δ49-57 fails to bind p58 of the wild type VacA and the reformation of VacA oligomers after acid exposure is blocked. Once again this raises the possibility that an oligomeric form of VacA might be required for its vacuolating cytotoxic activity.

VacA dominant negative mutant

In a recent study, using a FLAG-VacA toxin, which can be cleaved into the p37 and p58 domains, it has been demonstrated that the two fragments remain physically associated after proteolytic cleavage and were still able to form a VacA oligomeric structure (47). This suggests that there are two types of interactions between the two domains of VacA: intramolecular interactions between the p37 and p58 domains of an individual VacA monomer, and intermolecular interactions between p37 and p58 of different VacA molecules, necessary for the formation of oligomeric structures. As shown by the yeast two-hybrid system, deletion of 8 amino acids deletion in the p37 domain reduced the level of interaction with the p58 domain by 50 %. According to the model presented in figure10, we propose that p37 Δ49-57 lacks the capacity to interact with p58 of another monomer and that the region deleted is involved in the intermolecular interactions. Over the last 10 years, many studies have shown that due to the formation of a VacA anion selective channel, the toxin causes multiple effects on target cells *in vitro* (7,11-18), but the *in vivo* function of VacA and its relevance for *Helicobacter pylori* initial colonization of the stomach remains unclear (51). Since VacA Δ49-57 is produced and correctly secreted by *H. pylori* and has the capacity to enter epithelial cells without inducing a cytotoxic effect, we suggest that it could be used as a tool to understand the role of VacA in *H. pylori* infection.

VacA dominant negative mutant

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[Insert Acknowledgements here]

Abbreviations.

[1]The abbreviations used are: MALT, mucosa-associated lymphoid tissue; PCR, polymerase chain reaction; DMEM, Dulbelcco modified Eagle's medium; TCA, trichloroacetic acid; ONPG, *o*-Nitrophenyl-β-D-galactopyranoside

Figure Legends.

Fig. 1. **Construction of *H. pylori* SPM 326-Δ49-57 using a *sac*B-based counter-selection approach.** pBlueScriptKS p37, a plasmid containing a *vacA* fragment from *H. pylori* SPM 326 was mutated to introduce a *Nco*I site at the start codon. The *kan-sacB* cassette from pEnKSF (35) was introduced, after PCR amplification, into the *NcoI*/*Eco*NI site of pBlueScriptKS p37 to obtain pKan/sacB. The *kan-sacB* module contained on pKan/sacB was introduced into the genome of *H. pylori* SPM 326 by homologous recombination (A). The resulting strain was VacA$^-$ Km^r Suc^s. This mutant was transformed with pBS p37-Δ49-57, in which the nucleotides encoding amino acids 49-57 of VacA had been deleted (gray box). The *kan-sacB* cassette was removed by selecting for a second allelic-exchange event on the basis of sucrose resistance (B). The resulting strain was Km^s Suc^r and produced a mutated toxin (C). The amino acid numbering system used in this figure is based on designating the first amino acid (alanine) of the mature secreted toxin as amino acid 1.

Fig. 2. **Schematic representation of the *vacA* gene of *H. pylori* SPM 326-Δ49-57.**

Fig. 3. **Vacuolating activity of purified VacA and VacA Δ49-57.** Wild-type VacA and VacA Δ49-57 (35 μg/ml) were acid activated for 5 min at room temperature and then added to medium overlying HeLa cells for 7 h at 37°C. Vacuolating activity was quantified using a neutral red uptake assay.

VacA dominant negative mutant

Fig. 4. **Localization of the mutated toxin revealed by indirect immunofluorescence microscopy.** Non acid activated VacA Δ49-57 (5 μg/ml) was added to medium overlying HeLa cells. After 4 hours of infection, the toxin was stained with a mouse monoclonal anti-VacA antibody (C1G9) and a fluorescent labeled anti-mouse Ig secondary antibody. A, non permeabilized cells. B, permeabilized cells.

Fig. 5. **Structure analysis of VacA Δ49-57.** (upper panel) Transmission electron microscope visualization of purified toxin after quick freeze, deep etch preparation. A, a representative field showing a large number of molecules from the preparation of wild-type toxin. B, a VacA Δ49-57 toxin preparation. Bar 100 nm. (lower panel) Glycerol gradient sedimentation of concentrated culture supernatants at neutral and acidic pH. Concentrated supernatant from strains SPM 326 and SPM 326-Δ49-57 were centrifuged through 10-30% glycerol gradients. Gradients and samples were either at pH 7.5 or pH 3. Gradients were fractionated from the top and VacA was detected by Western-blot using rabbit polyclonal antibody.

Fig. 6. **Inhibition of wild-type VacA cytotoxic activity by VacA Δ49-57**. Acid activated VacA (10 μg/ml) was incubated with varying concentrations of VacA Δ49-57 acid activated (black) or not (grey) and then added to the medium overlying HeLa cells for 9 h at 37°C. Vacuolating activity was quantified using a neutral red uptake assay.

Results are expressed as percentage of neutral red uptake induced by the wild type toxin alone. Results represent the mean ± SD from three independent experiments.

Fig. 7. **Effect of VacA Δ49-57 on wild type VacA oligomerization.** The formation of oligomers was visualized by electron microscope after quick freeze, deep etch preparation and glycerol gradient sedimentation. (upper panels) A representative field showing molecules from the preparation of VacA and VacA Δ49-57 (A); acid activated VacA and VacA Δ49-57 (B) and acid activated VacA alone (C). Bar, 100 nm. (lower panels) 10-30% glycerol gradient sedimentation of the corresponding concentrated supernatants. Fractions were collected from the top and VacA was detected by Western-blot using a rabbit polyclonal antibody.

Fig. 8. **Interaction of VacA and VacA Δ49-57 *in vitro*.** Acid activated concentrated supernatant from wild type strain SPM 326 was incubated with increasing amounts of a SPM 326-Δ49-57 preparation before treatment with formaldehyde. For each ratio, formaldehyde-untreated concentrated supernatants were also incubated in the same conditions. Samples were analyzed by Western blotting using a rabbit polyclonal antibody. Expected size of the monomer (*), dimer (**) and trimer (***) of VacA are indicated.

Fig. 9. **Interaction between wild type p58 and p37 Δ49-57 in the yeast two hybrid system.** p37, p37 Δ49-57 and p58 fragments were cloned into plasmids encoding the transcription activation domain (pJG4-5L) and/or the DNA binding domain (pEG202-NLS) as described in Materials and Methods. Yeast cells were co-transformed with a combination of these plasmids as indicated and two hybrid interactions were quantified with a β-galactosidase liquid culture assay. β-galactosidase activity was expressed in Miller units. Results represent the mean ± S.D. of activity in three co-transformants assessed three different times.

Fig. 10. **Model of VacA inhibition reassembly in the presence of VacA Δ49-57 after acid exposure.** Acid exposed VacA oligomers dissociate into monomers and its p37 domain can interact with the p58 domain of the mutated VacA Δ49-57 cytotoxin leading to the formation of heterodimers. The 8 amino deletion of VacA Δ49-57 represented by the black cross disturbs the successive interaction with p58 necessary for obtaining the closing of the oligomer and leading to an unstable structure which has the tendency to dissociate. Wild type toxin is represented in blue whereas VacA Δ49-57 is colored in pink. For clarity, the p58 domain is represented without the p37 subunit (21).

VacA dominant negative mutant

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VacA dominant negative mutant

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Fig. 1

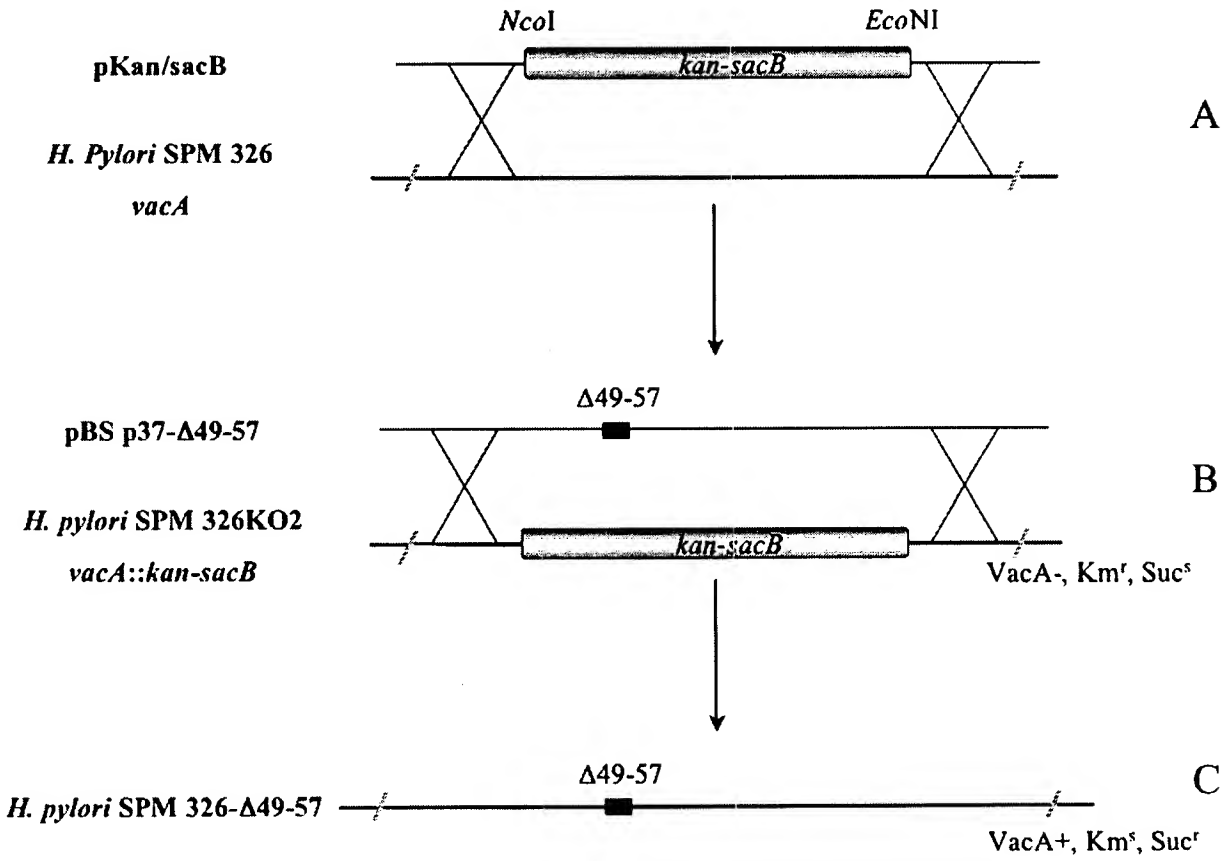
NcoI
EcoNI
pKan/sacB
kan-sacB
A
H. Pylori SPM 326
vacA
Δ49-57
pBS p37-Δ49-57
B
H. pylori SPM 326KO2
vacA::kan-sacB
kan-sacB
VacA-, Km^r, Suc^s
Δ49-57
C
H. pylori SPM 326-Δ49-57
VacA+, Km^s, Suc^r

Fig. 2

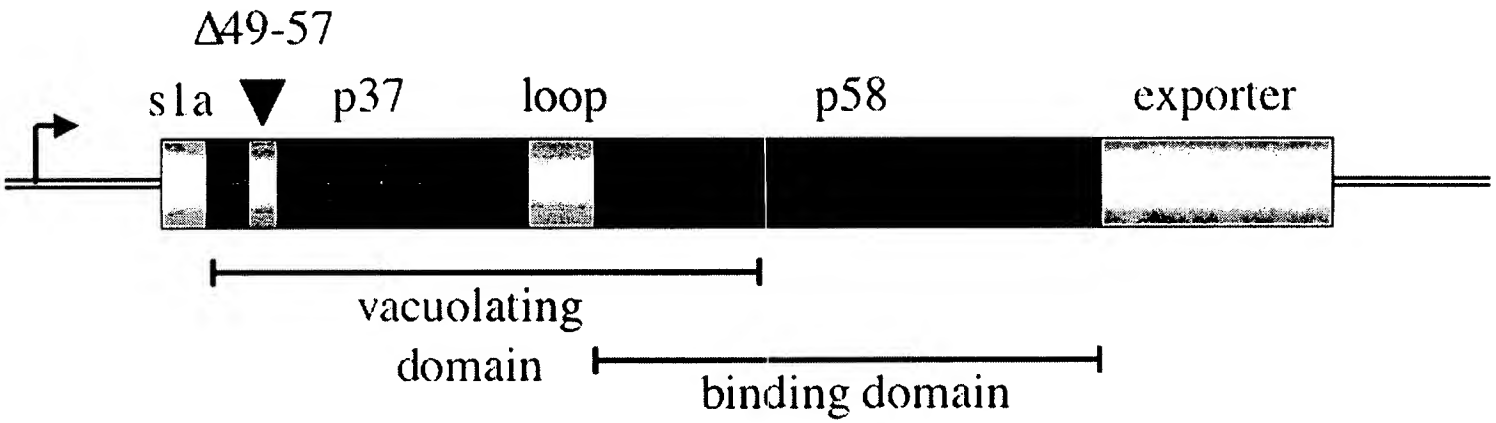
Δ49-57
s1a
p37
loop
p58
exporter
vacuolating
domain
binding domain

Fig. 3

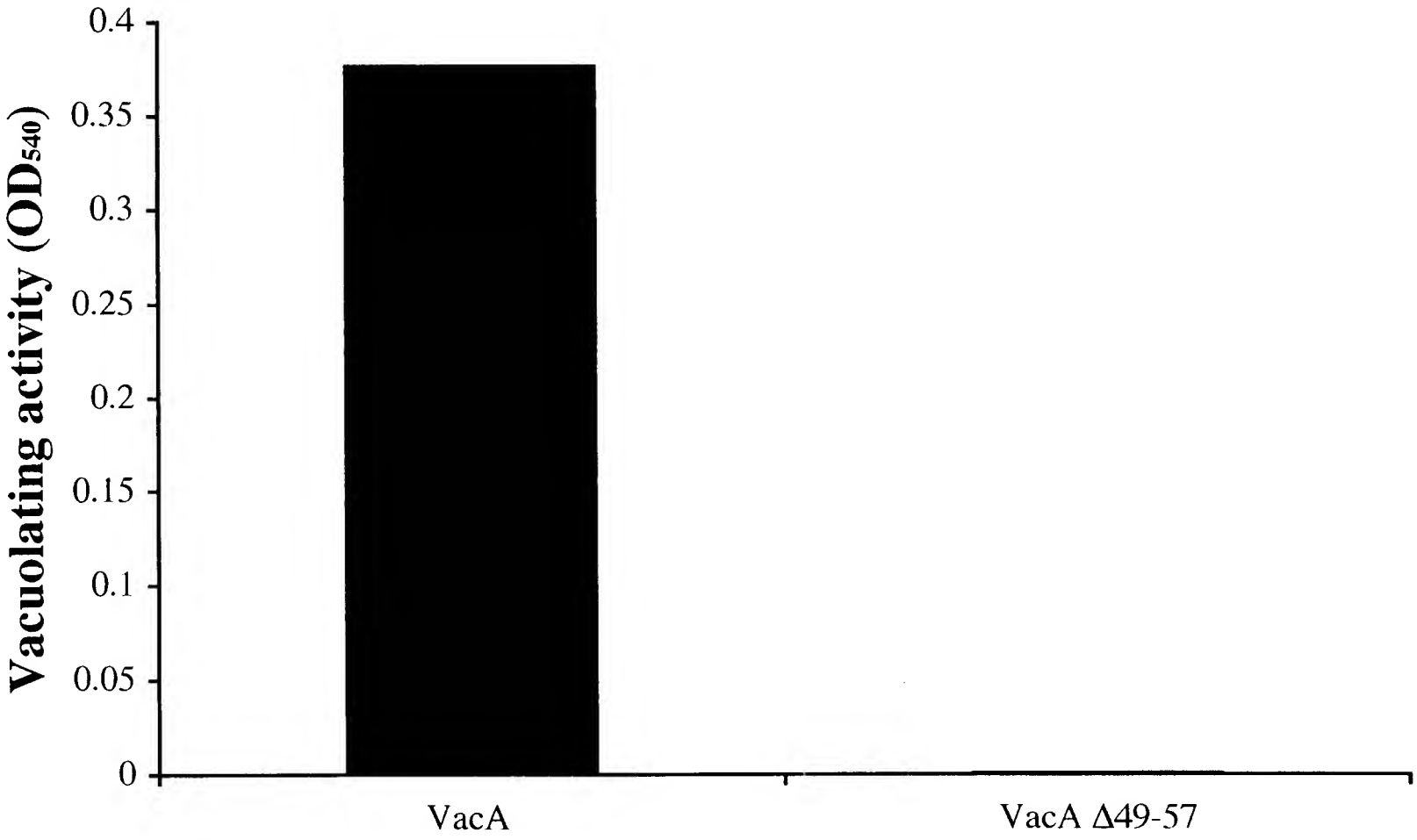
Vacuolating activity (OD_{540})
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
VacA
VacA Δ49-57

Fig. 4

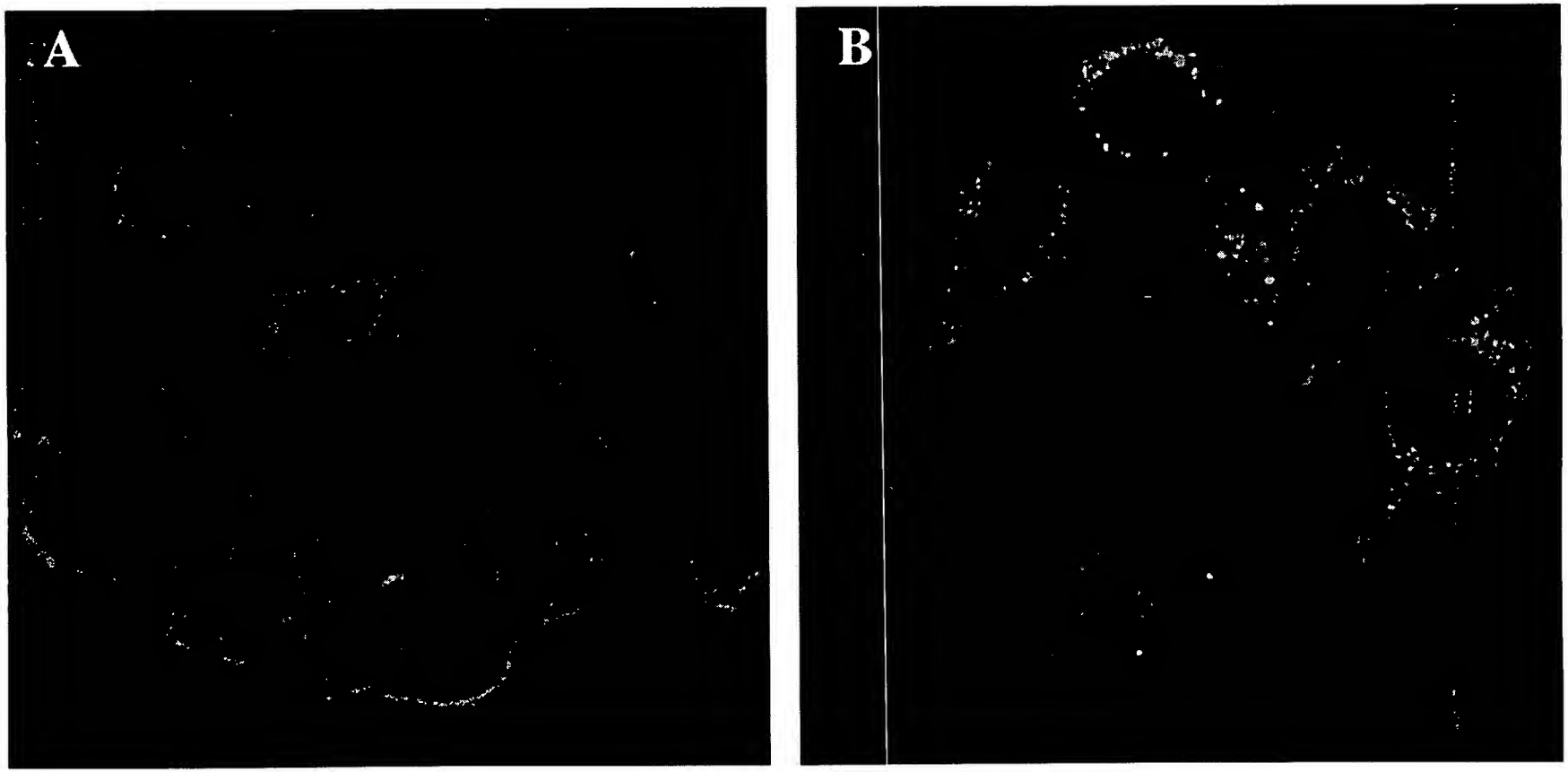

Fig. 5

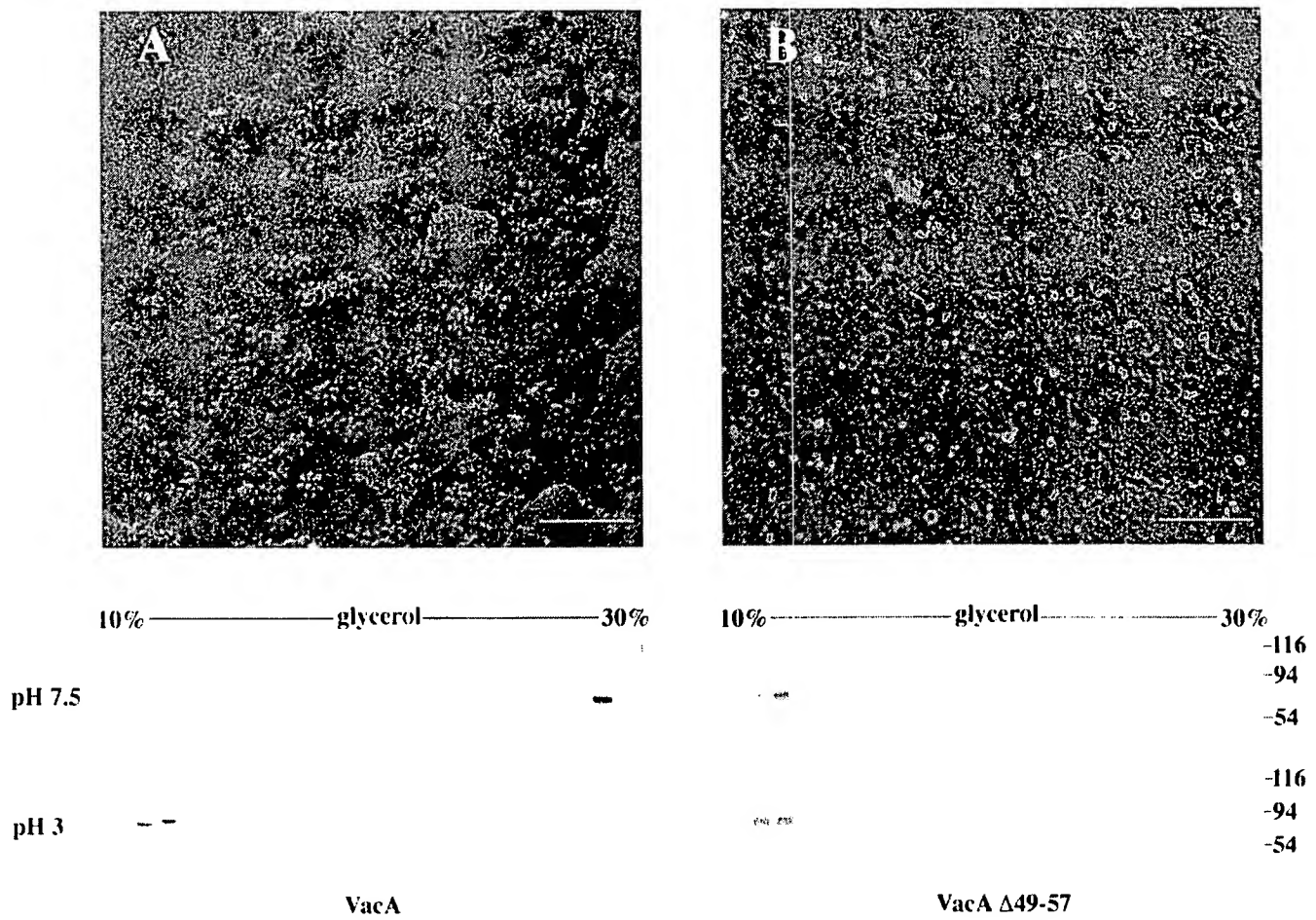
A
B
10%
glycerol
30%
10%
glycerol
30%
-116
-94
-54
pH 7.5
-116
-94
-54
pH 3
VacA
VacA Δ49-57

Fig. 6

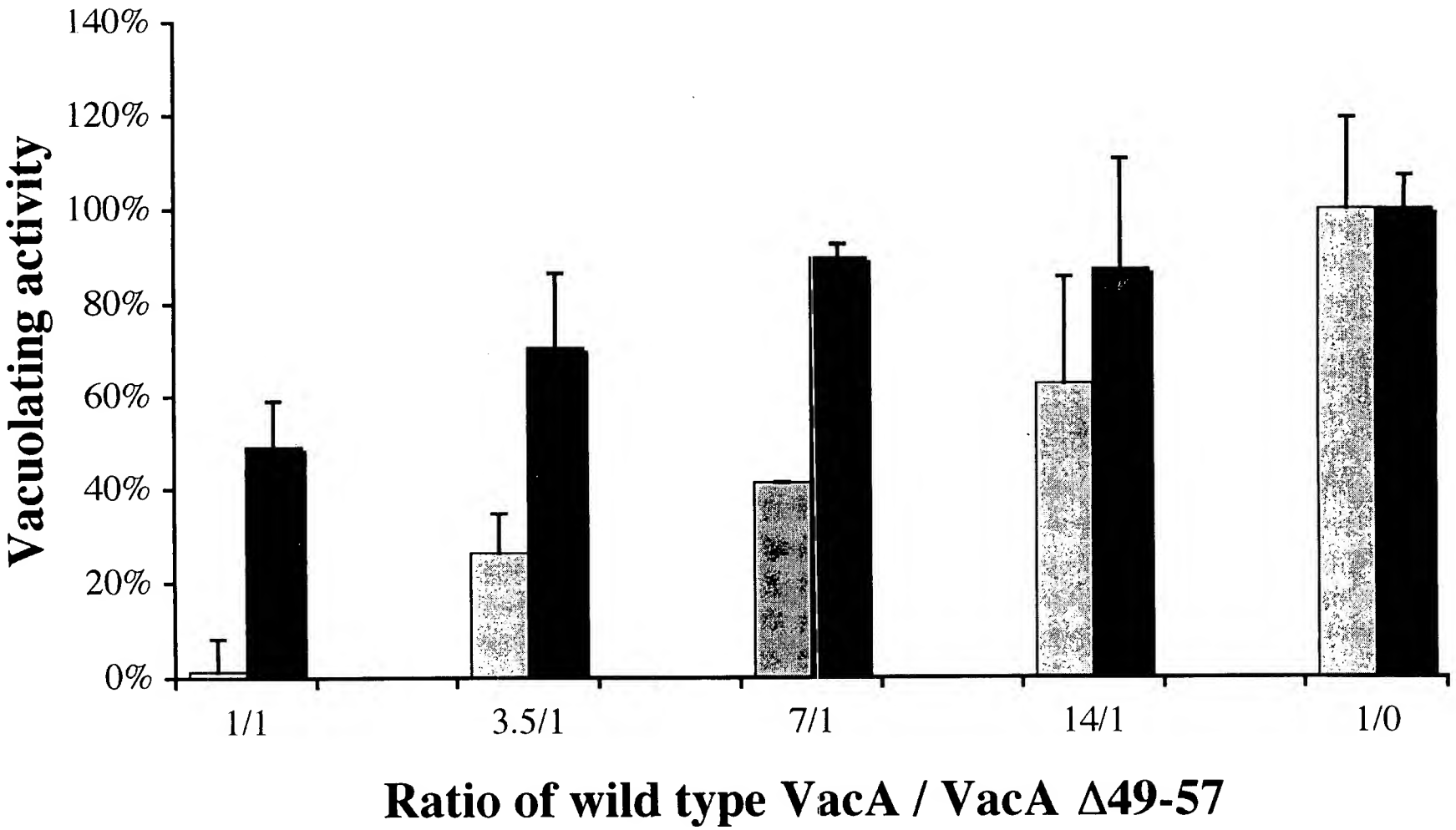
140%
120%
100%
80%
60%
40%
20%
0%
Vacuolating activity
1/1
3.5/1
7/1
14/1
1/0
Ratio of wild type VacA / VacA Δ49-57

Fig. 7

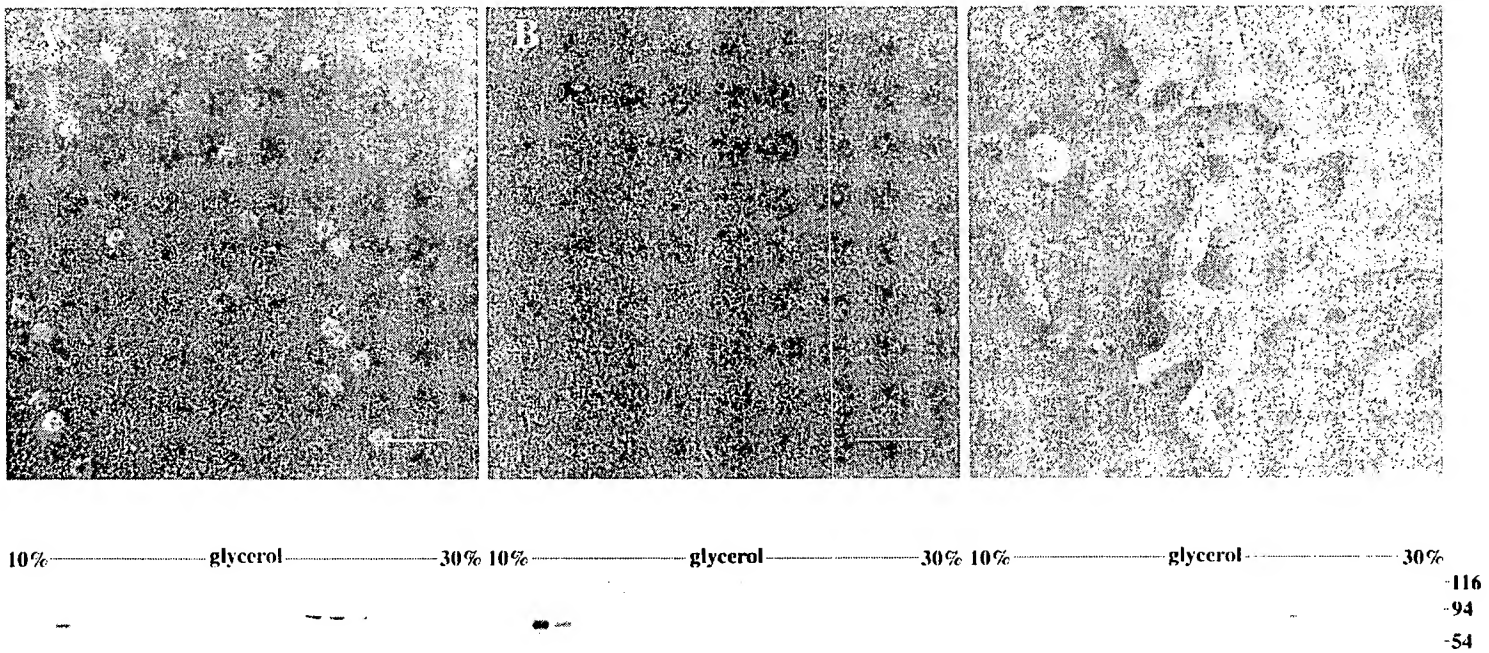
10%
glycerol
30%
10%
glycerol
30%
10%
glycerol
30%
116
94
54
VacA + VacA Δ49-57
Acid activated VacA + VacA Δ49-57
Acid activated VacA

Fig. 8

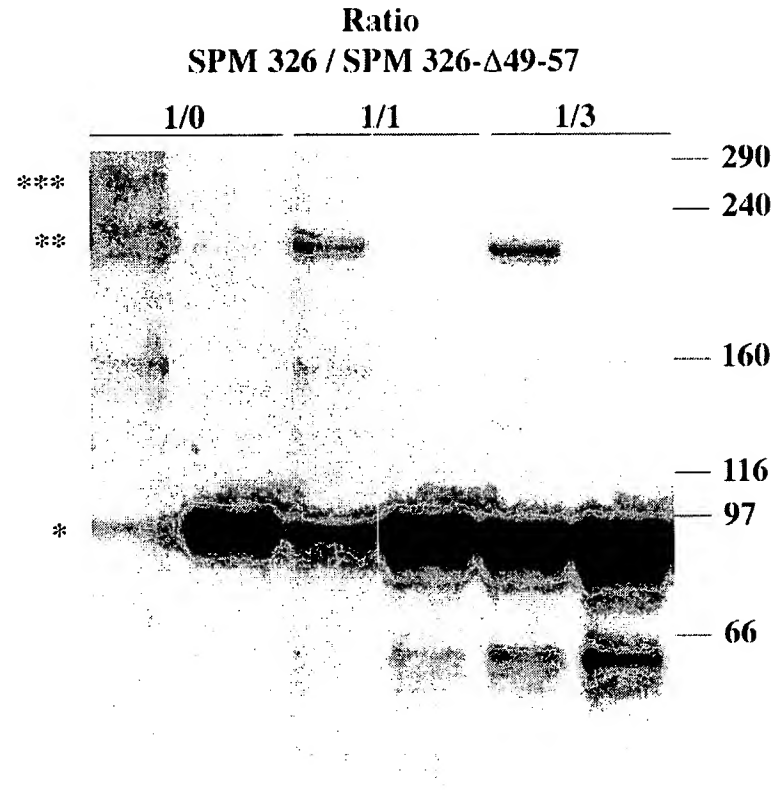
Ratio
SPM 326 / SPM 326-Δ49-57
1/0
1/1
1/3
290
240
160
116
97
66
***
**
*
Formaldehyde + - + - + -

Fig. 9

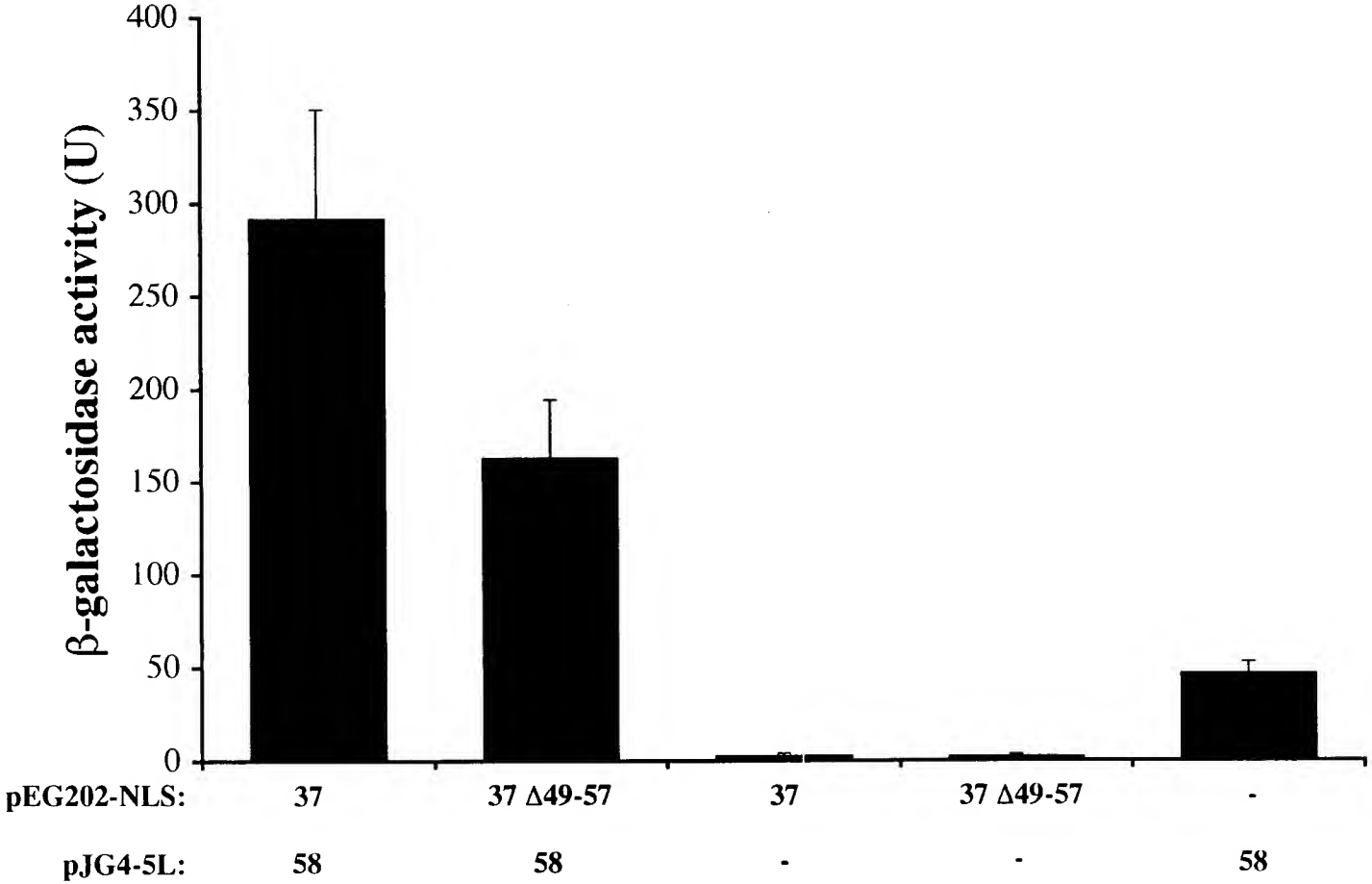
β-galactosidase activity (U)
400
350
300
250
200
150
100
50
0
pEG202-NLS:
37
37 Δ49-57
37
37 Δ49-57
-
pJG4-5L:
58
58
-
-
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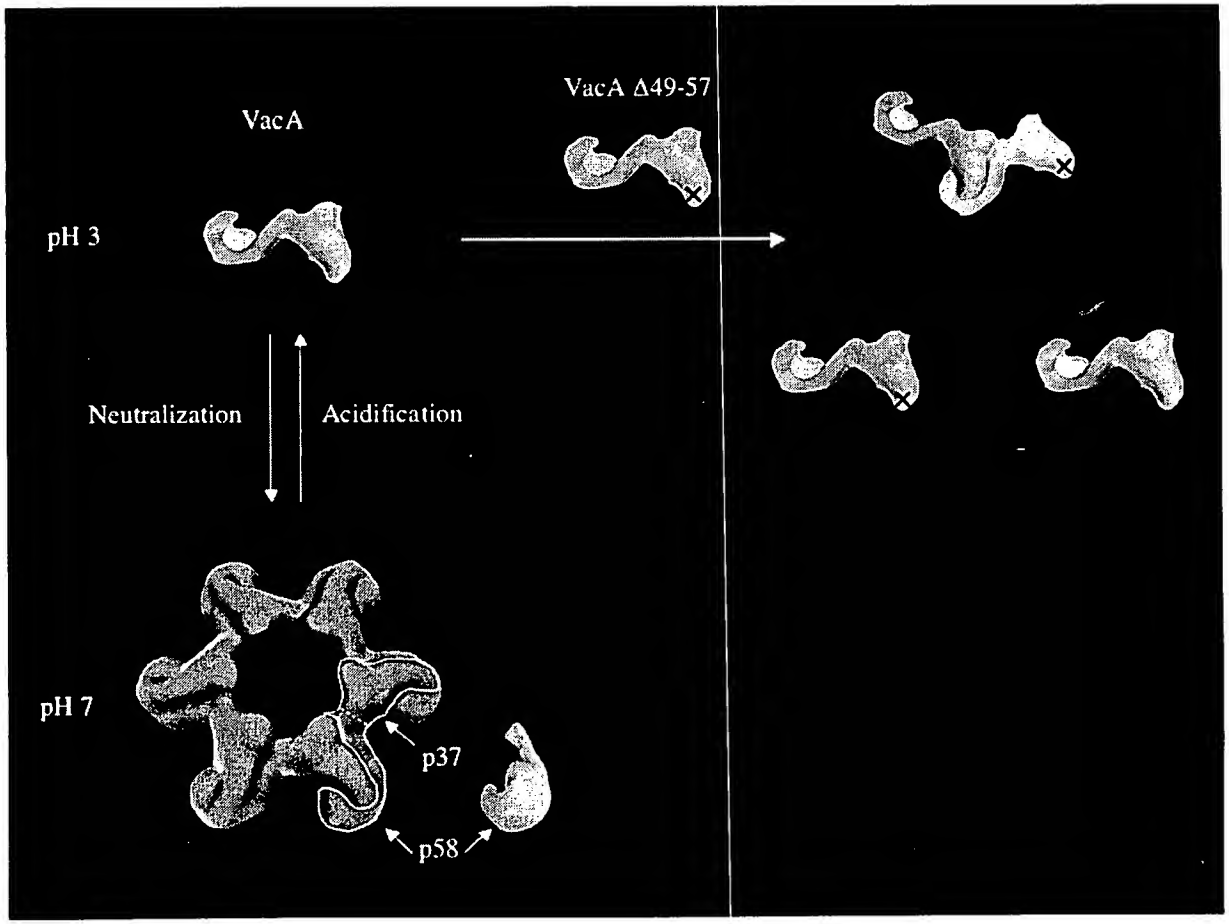
Fig. 10


VacA
VacA Δ49-57
pH 3
Neutralization
Acidification
pH 7
p37
p58

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3D Imaging of the 58 kDa Cell Binding Subunit of the *Helicobacter pylori* Cytotoxin

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Pathogenic strains of *Helicobacter pylori* produce a potent exotoxin, VacA, which intoxicates gastric epithelial cells and leads to peptic ulcer. The toxin is released from the bacteria as a high molecular mass homo-oligomer of a 95 kDa polypeptide which undergoes specific proteolytic cleavage to 37 kDa and 58 kDa subunits. We have engineered a strain of *H. pylori* to delete the gene sequence coding for the 37 kDa subunit. The remaining 58 kDa subunit is expressed efficiently and exported as a soluble dimer that is non-toxic but binds target cells in a manner similar to the holotoxin. A 3D reconstruction of the molecule from electron micrographs of quick-freeze, deep-etched preparations reveals the contribution of each building block to the structure and permits the reconstruction of the oligomeric holotoxin starting from individual subunits. In this model P58 subunits are assembled in a ring structure with P37 subunits laying on the top. The data indicate that the 58 kDa subunit is capable of folding autonomously into a discrete structure recognizable within the holotoxin and containing the cell binding domain.

Keywords: Helicobacter pylori; vacuolating cytotoxin; B-subunit; 3D reconstruction

Introduction

About 60% of the world population is chronically infected by *Helicobacter pylori*, a microaerophilic, Gram-negative bacterium that causes gastritis, peptic ulcer, MALT lymphoma and increases the risk of gastric cancer (Warren & Marshall, 1983; Parsonnet *et al.*, 1991). The bacterium, produces a secreted protein toxin which plays a key role in the gastric epithelial errosion and ulceration (Telford *et al.*, 1994a,b; Marchetti *et al.*, 1995; Cover, 1996; Labigne & de Reuse, 1996; Dunn *et al.*, 1997). The toxin, produced as a 140 kDa precursor, is exported as a 95 kDa polypeptide after cleavage of the C-terminal 45 kDa (Telford *et al.*, 1994a,b; Schmitt & Haas, 1994). The C-terminal domain shows homology with the C-terminal domain of a number of secreted proteins (Loveless & Saier, 1997), which is responsible for outer-membrane export through the autotransporter pathway (reviewed by Holland, 1998). The purified toxin observed by electron microscopy is a high molecu-

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Abbreviations used: VacA, vacuolating cytotoxin A; MALT, mucosa-associated lymphoid tissue; DMS, dimethylsuberimidate; EM, electron microscope; TER, trans-epithelial resistance; WT, wild-type; MCS, multiple cloning site; mAb, monoclonal antibody; Ig, immunoglobulin.

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lar mass regular flower-shaped oligomer with 6-fold or 7-fold radial symmetry (Lupetti *et al.*, 1996; Cover *et al.*, 1997; Lanzavecchia *et al.*, 1998). After release from the bacterium each monomer can be cleaved at a specific hydrophilic loop into two fragments of 37 kDa and 58 kDa that remain associated in the oligomeric structure, suggesting that they may represent two distinct cytotoxin subunits (Telford *et al.*, 1994a; Lupetti *et al.*, 1996); however, the location of the subunits in the holotoxin is not yet described.

Prior to exerting its toxic activity, VacA binds to a specific receptor (Yahiro *et al.*, 1997; Massari *et al.*, 1998) and is internalized by the cell (Garner & Cover, 1996; Massari *et al.*, 1998). DNA transfection experiments have shown that VacA acts in the cytosol, since intracellular expression of the *vacA* gene resulted in full vacuolating activity (de Bernard *et al.*, 1997). Vacuolating activity is the result of altered intracellular vesicle trafficking, which leads to the accumulation of hybrid compartment containing markers of late endosomes and lysosomes (Molinari *et al.*, 1997). Vacuolation requires the activity of the small GTP binding protein Rab7 (Papini *et al.*, 1997). Moreover, VacA has been shown to impair the degradative properties of the endocytic pathway in HeLa cells (Satin *et al.*, 1997) and antigen presentation by B-cells by preventing processing and maturation of antigens (Molinari *et al.*, 1998).

These data suggest that VacA may have similarities with the AB family of dichain toxins in which the B or binding moiety is involved in cell interaction and translocation of the A or active moiety into the cytoplasm of the cell. However, so far little is known about the structure of the domains of the VacA molecule and their function. Recently some progress in understanding the function of the P58 and the P37 moieties has been made. An allelic variant of VacA (m2), which differs extensively in an approximately 300 amino acid segment of the p58 domain, failed to bind and to intoxicate HeLa cells, indicating a role for this domain in cell binding (Pagliaccia *et al.*, 1998). Interestingly, the m2 variant bound to and intoxicated another epithelial cell line, suggesting recognition of different receptors by the two isoforms. Furthermore, transfection of HeLa cells with a gene coding for the P37 from which most of the sequence coding for the P58 subunit had been deleted induced vacuole formation, suggesting that the P37 subunit is responsible for the vacuolating activity (de Bernard *et al.*, 1998).

We have modified the *vacA* gene in a toxigenic strain of *H. pylori* to express only the putative B subunit (P58) of the vacuolating cytotoxin. The P58 moiety was expressed and secreted in the supernatant as a soluble molecule independently of the P37 subunit. Moreover, purified P58 bound eukaryotic cells but was not endocytosed and did not cause intoxication. Finally, electron microscopy and 3D image reconstruction of quick-freeze, deep-etch preparation of the P58 replicas, permitted the reconstruction of the global architecture of the P58 molecule and to propose a model of the wild-type holotoxin.

Results

The P58 domain is produced and exported correctly

Attempts to produce active recombinant VacA in *Escherichia coli* expression systems have failed. The produced protein invariably formed inclusion bodies of insoluble protein and after solubilization was recognized only poorly by antisera raised against the native protein indicating incorrect folding (Manetti *et al.*, 1995; Massari *et al.*, 1998). Hence, in order to study the structure and function of the individual subunits we engineered the gene coding for VacA in an *H. pylori* strain to express only the 58 kDa (P58) subunit (Figure 1). To achieve this, we used the restriction sites *Eco*RI and *Eco*NI to construct a plasmid containing the *vacA* gene lacking the sequence coding for most of the P37 subunit (amino acid residues 91 to 330). This construct (*vacAP58*) carries sequences coding for

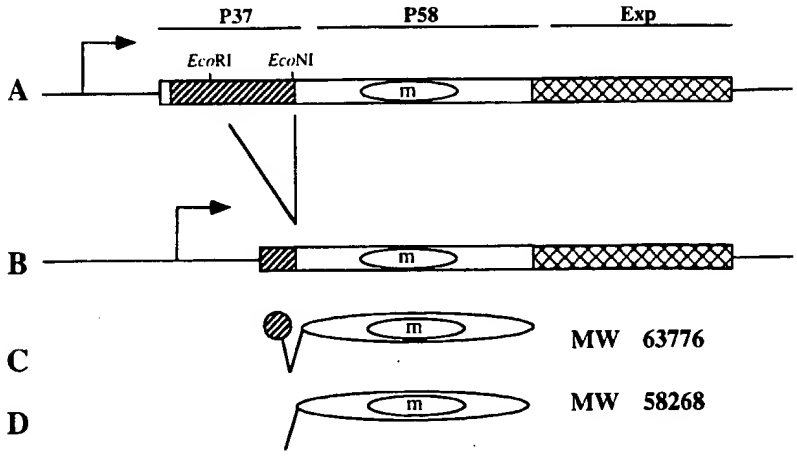

Figure 1. Schematic representation of the wild-type *vacA* gene (a), the *in vitro* constructed *vacA P58* gene (b), the expected product of the *vacA P58* gene (c), and the product obtained (d).

the signal peptide, 66 residual amino acids of P37, the full P58 domain and the C-terminal domain required for outer-membrane export (Loveless & Saier, 1997). This construct was introduced into an *H. pylori* strain in which the *vacA* gene had been interrupted with a cassette carrying a kanamycin resistance gene and the *sacB* counter-selectable marker (Copass *et al.*, 1997). Clones in which the cassette had been substituted with the *vacAP58* construct (*H. pylori* SPM 326-P58) were selected by growth in sucrose and sensitivity to kanamycin.

In order to check the expression of the *vacAP58* construct, water-extracts from wild-type *H. pylori* SPM 326 and from the mutant *H. pylori* SPM 326-P58 were prepared and analysed by Western-blot using a polyclonal antiserum which recognizes both subunits of VacA (Telford *et al.*, 1994b). As expected, in the water-extract of the wild-type *H. pylori* SPM 326, we detected the 95 kDa polypeptide corresponding to VacA monomer while in the case of *H. pylori* SPM 326-P58, we detected a band with an apparent molecular mass of approximately 60 kDa (Figure 2(a)).

Immunoblot analysis of bacterial pellet and supernatant revealed that the P58 molecule was released in the supernatant as efficiently as the wild-type VacA (Figure 2(b)). The apparent molecular mass of approximately 60 kDa was compatible with the predicted molecular mass of the construct (63,776 Da). The absence in the culture supernatant of cytoplasmic contaminants due to bacterial lysis was confirmed by demonstrating the absence in this fraction of σ^{80}, a marker of cytoplasmic proteins (Figure 2(c)). We conclude that the P58 molecule is produced, correctly processed and efficiently exported in a soluble form to the bacterial supernatant, suggesting a structural autonomy for this domain.

The P58 molecule was purified from a culture supernatant from *H. pylori* SPM 326-P58 using a recently described immuno-affinity procedure (Reyrat *et al.*, 1998). The amino-terminal sequence of the purified material was found to be G-K-G-F-N, corresponding to amino acid 87 to 91 in the VacA protein. This showed that in addition to the signal peptide, most of the remaining P37 has been proteolitically removed leaving the entire 58 kDa subunit plus 27 additional amino acid residues at the amino terminus (Figure 1(d)). The calculated mass of this P58 molecule is 58,268 Da. The P58 molecule was recognized by a monoclonal antibody, G1G9, directed against a conformational epitope (Reyrat *et al.*, 1998), suggesting at least a partially correct folding for this subunit (data not shown).

The P58 molecule forms a dimer

The purified P58 molecule was observed by quick-freeze, deep-etch electron microscopy. In this procedure, the protein is first adsorbed onto mica flakes, flash-frozen and shadowed with carbon and platinum. The extremely rapid freezing prevents the distortion of the molecules which may occur using other techniques and allows the mold to represent accurately the surface of the molecule. The electron micrographs show that the majority of the purified material is structurally homogeneous with molecule replicas having a small rod shape of about 21 nm x 9 nm in size (Figure 3(a)). In comparison, the holotoxin molecule appears as a flower-like structure with a diameter of about 30 nm. Images of P58 subunits were aligned and processed by statistical classification and image averaging (Lanzavecchia *et al.*, 1998). The resulting image revealed that the molecule has dyad symmetry and that each half of the molecule has a curved appearance remarkably similar to the petals of the intact oligomeric toxin (see boxed monomers in Figure 3(b)).

All images appeared with the same handedness and statistical analysis was not able to evidence any group of molecules with opposite chirality. This suggests a strong interaction between the negatively charged mica surface and one face of the P58 molecule, as described for the WT oligomers (Lanzavecchia *et al.*, 1998). This feature makes the sample suitable for a random conical tilt reconstruction of the replicas (Radermacher *et al.*, 1987; Lanzavecchia *et al.*, 1998). According to this strategy of electron tomography (Frank, 1992) the 3D map of the replica is computed by using pairs of micrographs portraying the same field at 0° and 45° tilt angles. The reconstructions produced emphasize

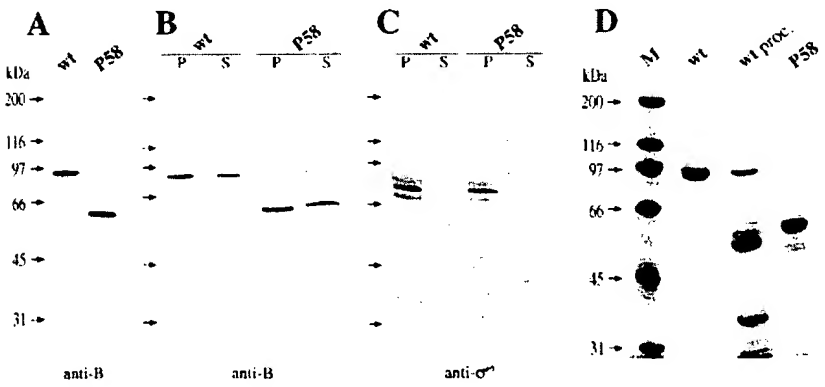

Figure 2. (a) Immunoblot of denaturing SDS-PAGE of VacA and P58 in water-extracts of *H. pylori* SPM 326 and of *H. pylori* SPM 326-P58. (b) Immunoblot of denaturing SDS-PAGE of localization of VacA and P58 in *H. pylori* SPM 326 and in *H. pylori* SPM 326-P58. P, pellet, S, supernatant. (c) Immunoblot of denaturing SDS-PAGE of localization of σ^{80} in *H. pylori* SPM 326 and of *H. pylori* SPM 326-P58. (d) Coomassie blue staining of purified VacA, processed VacA, and P58.

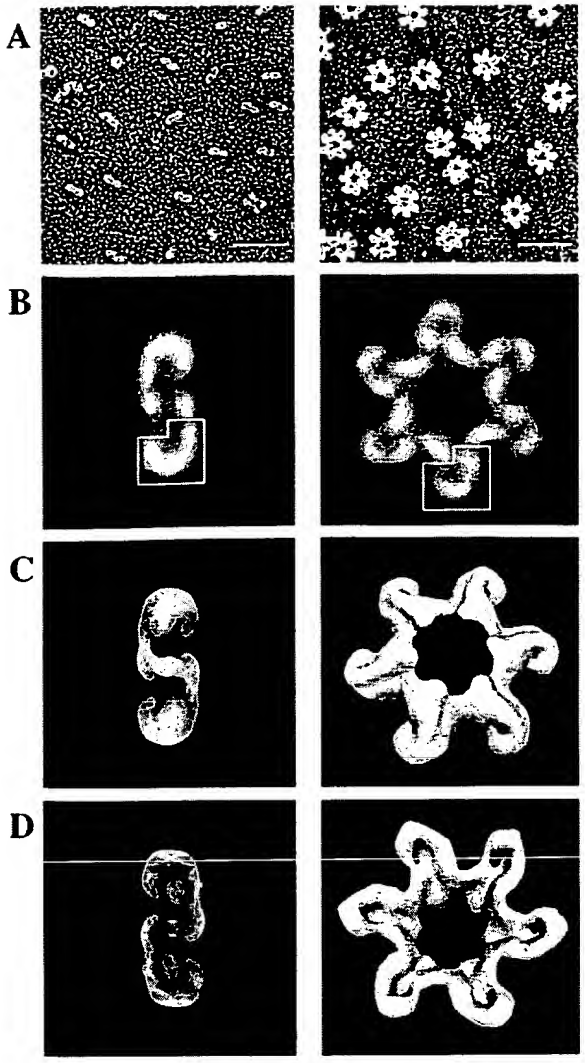

Figure 3. (a) Electron micrograph of purified VacA and P58. The bar represents 50 nm. (b) Average images of aligned molecules. (c) 3D top views of the reconstructions of the mold. (d) 3D bottom views of the reconstructions of the mold. Left panel, P58; right panel, WT VacA.

considerably the similarity in structure between the halves of the p58 molecules and the petals of the holotoxin (Figure 3(c)).

In the reconstructions, also the lower face of the metal mold can be visualized, revealing the volume occupied by the molecule (Figure 3(d)). Moreover, 3D reconstruction allowed us to have a precise estimation of the dimensions of the molecule, and to compare these data with the analogous reconstruction of the intact toxin. After subtraction of the metal coal, the P58 complex is about 15 nm × 4 nm whereas the diameter of the intact toxin is 24 nm. The height of the molecules, 5-6 nm, is similar to that of the peripheral arms of the intact oligomer (see Lanzavecchia *et al.*, 1998). The handedness of the P58 molecule coincides with that of the intact oligomer, suggesting that P58 subunits are arranged to form the radial arms of the molecule. However, the dimensions of the molecules observed are too large to be accommodated within the holotoxin structure. In fact, the overall length of the P58 molecules is about twice that of the intact arms of the holotoxin (Lanzavecchia *et al.*, 1998). This and the dyad symmetry of the molecules suggest strongly that the P58 molecules observed on the Mica are dimers of the 58 kDa subunit.

Efforts to confirm the dimeric nature of the P58 in solution by electrophoresis or gel filtration in the absence of denaturation were thwarted by the tendency of the molecule to interact with the support, which resulted in retarded and anomalous migration (data not shown). However, mild cross-linking with the bivalent reactive compound DMS (dimethylsuberimidate), resulted in the appearance of a high molecular mass species that appears as a smear between 120 and 150 kDa in denaturing polyacrylamide gel electrophoresis (Figure 4(a)). The observed molecular mass after cross-linking strongly suggests the presence of a dimer. The smear is likely to be due to incomplete denaturation due to the cross-linking within the molecule, as otherwise discrete bands corresponding to multiples of the 58 kDa would be expected. There was no increase in the high molecular mass material observed even at fivefold higher concentration of cross-linker (data not shown), indicating that cross-linking occurred between preformed dimers.

These data were confirmed by a mass determination of a cross-linked sample. Figure 4(b) clearly shows the presence of three signals of *m/z* of 30,556, 61,145 and 123,329 in the mass spectrum. These values correspond well with those calculated for the double and single protonated form of the P58 monomer and to the dimeric form of the P58 molecule. As calibration was done using a single external standard, the error in mass is expected to be within 300-400 Da. In addition, the number of molecules of cross-linker (M_W 241) contributing to the mass is unknown. Only a single sharp peak was observed at the position of the dimer, confirming that the smear observed in SDS-PAGE was due to cross-linking in different conformations. No material whatever was detected at *m/z* ratios higher than that corresponding to the dimer. In the absence of cross-linker, the peak corresponding to the dimer (*m/z* 117,486) was, as would be expected from the strong chemical conditions of the assay, very much reduced compared to the two major signals of *m/z* 29,223 and 58,862 corresponding, respectively, to the double and single protonated form of the P58 monomer (Figure 4(c)). The mass of the P58 molecule in the absence of the cross-linker was very similar to the calculated mass (58,662/58,268), thus confirming that the processing site at the C terminus is at or within a few amino acid residues of that of the wild-type VacA.

We conclude from these data, taken together, that the structures observed in the EM are of

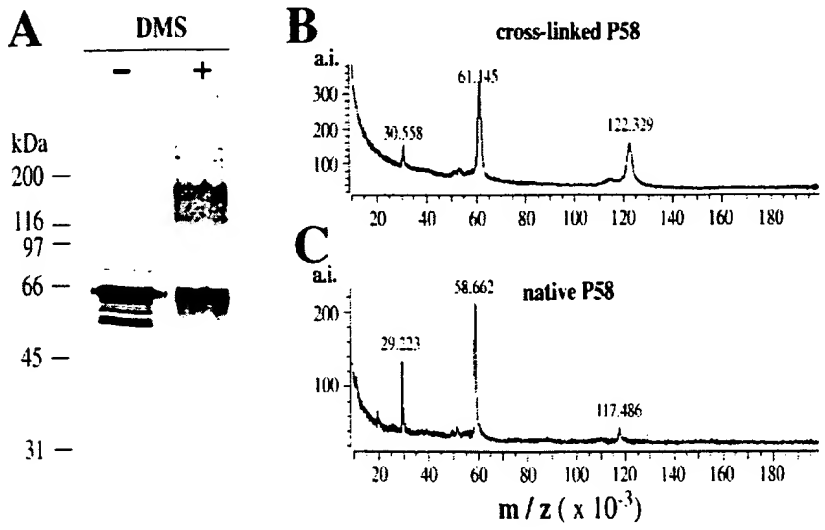

Figure 4. (a) Western blot analysis of P58 or P58 incubated with 2 mg/ml (final concentration) DMS as described in Materials and Methods. (b) Maldi-tof analysis of the cross-linked P58 material. (c) Maldi-tof analysis of P58 not treated with cross-linker.

dimers of the 58 kDa subunit and that each half of the dimer corresponds to the peripheral petal structure observed in the reconstructions of the oligomeric holotoxin.

The P58 domain interacts with the surface of target cells

Binding of P58 to target cells was assessed by indirect immunofluorescence and flow cytometry. As seen in Figure 5, the purified P58 molecule gave saturable binding with a dose response similar to the wild-type VacA, indicating that even in the absence of the 37 kDa subunit, P58 has a conformation still capable of interacting with the cell. It should be noted that a recombinant form of VacA, which does not fold correctly, failed to bind target cells (Massari *et al.*, 1998). Binding was confirmed by immunoflourescence microscopy. As seen in Figure 6(a), cells treated by P58 at 0 °C were labelled at the level of the plasma membrane with an anti-VacA polyclonal serum, demonstrating that P58 is able to interact with the surface of the target cell. In contrast to the intact VacA, the P58 moiety was, however, not internalized after binding as cytoplasmic fluorescence could not be detected in cells incubated with P58 at 37 °C for four hours (Figure 6(b) and (c)). Cells treated with the wild-type toxin and probed with the C1G9 mAb, which recognizes a conformational epitope localized in the P58 moiety (Reyrat *et al.*, 1998), gave a very clear cytoplasmic signal, demonstrating unambiguously that in the context of the intact oligomer, the P58 subunit was efficiently internalized (Figure 6(d)). These results suggest that either the P37 subunit is required for cell entry or that the hexameric structure may be important for a productive interaction with the cell.

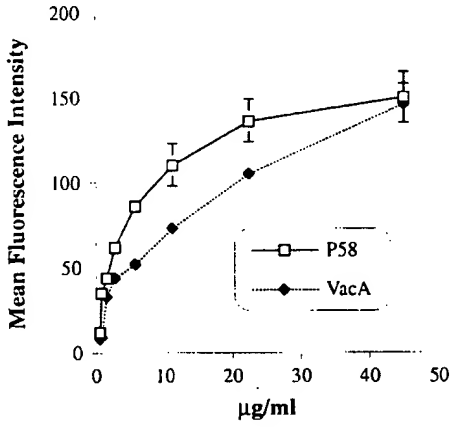

Figure 5. Binding of VacA and P58 to RK-13 cells. Cells were incubated at 0 °C with increasing concentrations of VacA or P58 and the binding was revealed by flow cytometry using ml specific antiserum and FITC-labelled anti-rabbit Ig. VacA (□), P58 (■). MFI, mean fluorescence intensity.

P58 is devoid of cytotoxic activity

The *H. pylori* SPM 326-P58 strain was assessed for cytotoxicity in the well characterized HeLa cell assay. Water-extracts were prepared and the extent of vacuolation was quantified using the neutral red uptake method (Cover & Blaser, 1992). As seen in Figure 7(a), water-extract from the wild-type strain (*H. pylori* SPM 326) induce a significant neutral red uptake, whereas water extract of the *H. pylori* SPM 326-P58 strain did not induce any significant neutral red uptake. The level of neutral red uptake induced by a water-extract of the *H. pylori* SPM 326-P58 was similar to an extract of an isogenic strain in which the *vacA* gene had been interrupted, thus demonstrating that this molecule was devoid of any cytotoxicity.

It has been shown that the *H. pylori* strain expressing the VacA cytotoxin possesses the ability to induce a decrease in the trans-epithelial resistance (TER) of a polarized epithelial cells monolayer

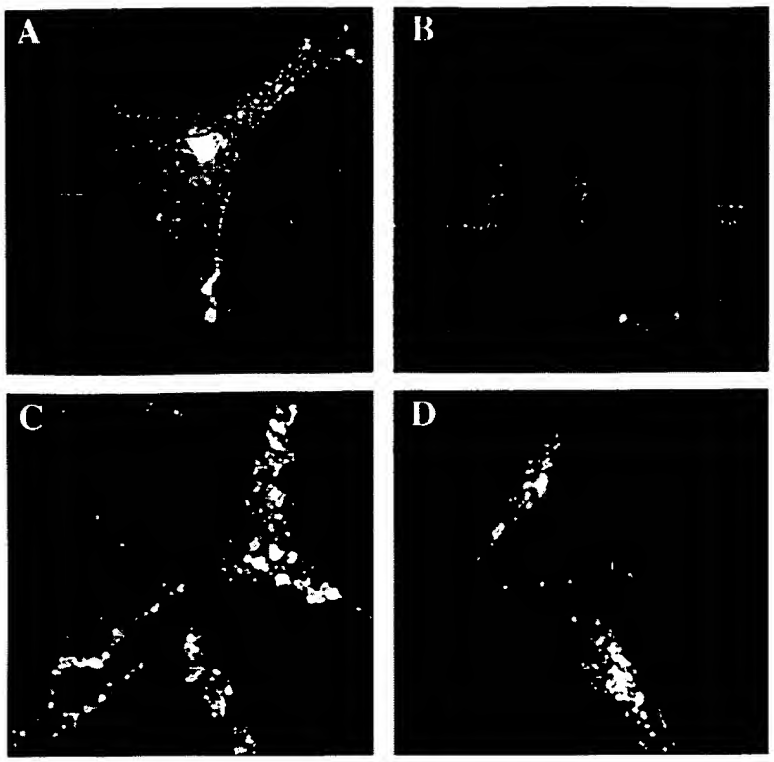

Figure 6. (a) Binding of P58 to HeLa cells revealed by indirect immunofluorescence microscopy using rabbit polyclonal immunoglobulin. (b) Lack of intracellular staining of P58 in HeLa cells. (c) Control of internalization of native VacA by HeLa cells detected after membrane solubilization. (d) Detection of intracellular 58 kDa subunit with P58 specific mAb (C1G9) in cells treated with native VacA.

(Papini *et al.*, 1998; Pelicic *et al.*, 1999). Such activity was investigated for *H. pylori* SPM 326-P58. We found that this strain, like the *vacA*$^-$ knock-out strain (*H. pylori vacA::Km*), was devoid of any TER activity, while the WT strain was able to induce a clear decrease of TER (Figure 7(b)).

To exclude that the lack of activity of the P58 molecule was not due to its inability to be internalized, HeLa cells were transfected with a construct encoding either the WT VacA or the P58 molecule and the extent of vacuolation was quantitated using the neutral red uptake. After four hours of expression, massive vacuolation was observed in the case of the construct encoding the wild-type molecule but not in the case of the construct encoding the P58 molecule, even after longer expression time (Figure 7(c)). However, immunofluorescence microscopy showed that the P58 molecule was expressed intracellularly (data not shown).

These results demonstrate that the construct encoding the P58 molecule is devoid of cytotoxic activity, as assessed by both TER and vacuolation assays whether added extracellularly or when expressed intracellularly in eukaryotic cells. Thus, it is very likely that the P37 molecule carries the region responsible for cell vacuolation.

Discussion

Many bacterial protein toxins are arranged in structural domains corresponding to distinct functions. In the AB family of dichain toxins, the A moiety, which contains the catalytic activity, is associated with an independently structured B moiety responsible for receptor interaction and translocation to the cytoplasm. The A and B subunits may be expressed as part of the same precursor polypeptide as is the case for diphtheria toxin, or they may be the products of independent genes as is the case for the heteropentameric B moiety of pertussis toxin (Domenighini *et al.*, 1995). One characteristic of these domain structures is that they are frequently capable of folding correctly in isolation from the other subunit. Here we have shown that the 58 kDa product of natural proteolysis of VacA can be efficiently expressed and secreted as a soluble molecule by *H. pylori* independently of the 37 kDa fragment, suggesting an independent folding and also an independent function. The compact structure of the P58 domain was also supported by the proteolytic removal of the 54 amino acid residues, corresponding to part of the P37 subunit, which remain in the truncated construct and its interaction with a mAb recognizing a conformational epitope in the native molecule. It is worth noting that a similar domain expressed in an heterologous context, in *E. coli*, give place to inclusion bodies, and the solubilized protein neither bound to target cells nor was recognized by mAb C1G9, thus suggesting missfolding (Manetti *et al.*, 1995; Massari *et al.*, 1998).

Interestingly, the P58 molecule forms a dimeric structure in solution. This may reflect the interaction, in the intact molecule, involved in holding together the oligomeric structure. However, it is difficult to understand why the P58 domain should

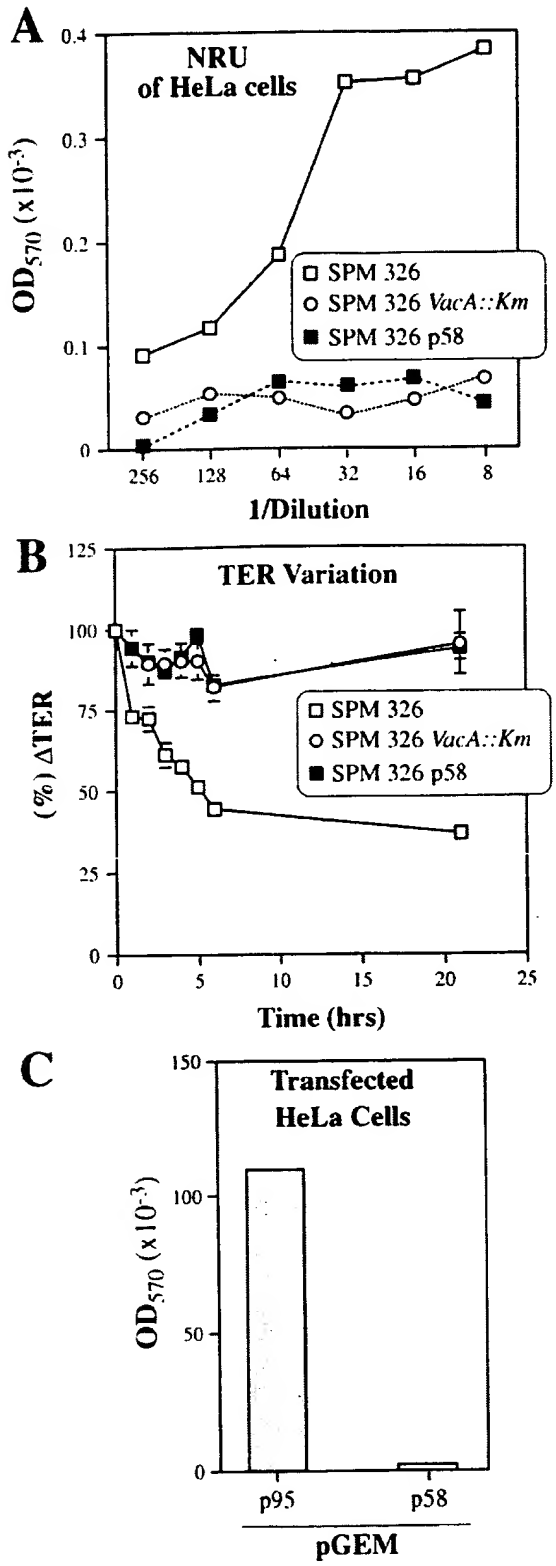

Figure 7. (a) Vacuolation of HeLa cells in response to water-extracts of *H. pylori* SPM 326 (□), *H. pylori* SPM 326 *vacA::Km* (○), or *H. pylori* SPM 326-P58 (■) as determined by the neutral red uptake assay. (b) Decrease in trans-epithelial resistance (TER) of MDCK cells in response to *H. pylori* SPM 326 (□), *H. pylori* SPM 326 *vacA::Km* (○), or *H. pylori* SPM 326-P58 (■). (c) Neutral red uptake of HeLa cell transfected with pGEMp95 (*vacA*) or with pGEMp58 (*vacA P58*).

form dimers rather than hexamers. On the other hand, the dimerization may be due to interaction between hydrophobic surfaces normally masked in the intact molecule. One possibility is that this region is involved in the interaction between the 58 kDa and the 37 kDa subunits which remain associated in the oligomeric structure even after cleavage of the connecting loop. This hypothesis is supported by observation of the 3D reconstructions of the replicas of the P58 and of the intact oligomer. The P58 molecule has a striking similarity with the peripheral arms of the oligomer. Furthermore, the dimensions of the analogous structure fit perfectly with a model in which the 37 kDa subunit forms a ring structure on top of and at the centre of a base formed by the 58 kDa subunit. This model is illustrated in the reconstruction shown in Figure 8. In Figure 8(a), the structure of half of the P58 dimer has been arranged in a ring, corresponding to the diameter of the intact oligomer. In Figure 8(b), a section of the upper part of the oligomer shown in Figure 8(c) has been superimposed on the ring of P58 molecules to bring the height up to that of the intact oligomer. The two parts match each other perfectly and the resulting reconstruction is almost identical with the 3D reconstruction of the native toxin both in appearance and in overall dimensions (compare Figure 8(b) with (c)). The model gives a clear idea of the arrangement of the P37 subunits with respect to the P58 subunits.

From the above reconstruction, it is not clear, however, which P37 and P58 subunits belong to the same 95 kDa monomer. Individual monomers may be simply inserted in the oligomer like wedges of a pie. However, we favour a model in which the monomers are intercalated with each other to form the ring structure in such a way that the 37 kDa subunit of one monomer lies on top of the 58 kDa subunit of the adjacent monomer, as shown in Figure 9. Thus, both P37 and P58 subunits would contribute to the formation of the structure. In support of this model, the putative 37 kDa subunit appears to lie orientated obliquely with respect to the underlying 58 kDa subunit and appears to traverse two monomers (best seen in the average image in Figure 3(b)). Furthermore, shortening of the flexible loop between the 37 kDa and 58 kDa subunits constrains the molecule to form only hexamers rather than a mixture containing predominantly heptamers (Burroni *et al.*, 1998). Hence pulling the 37 kDa and 58 kDa subunits closer together would reduce the flexibility of the interaction and the number of monomers which could enter into the structure. This model may also explain why the P58 subunit fails to form higher oligomers in the absence of the 37 kDa subunit.

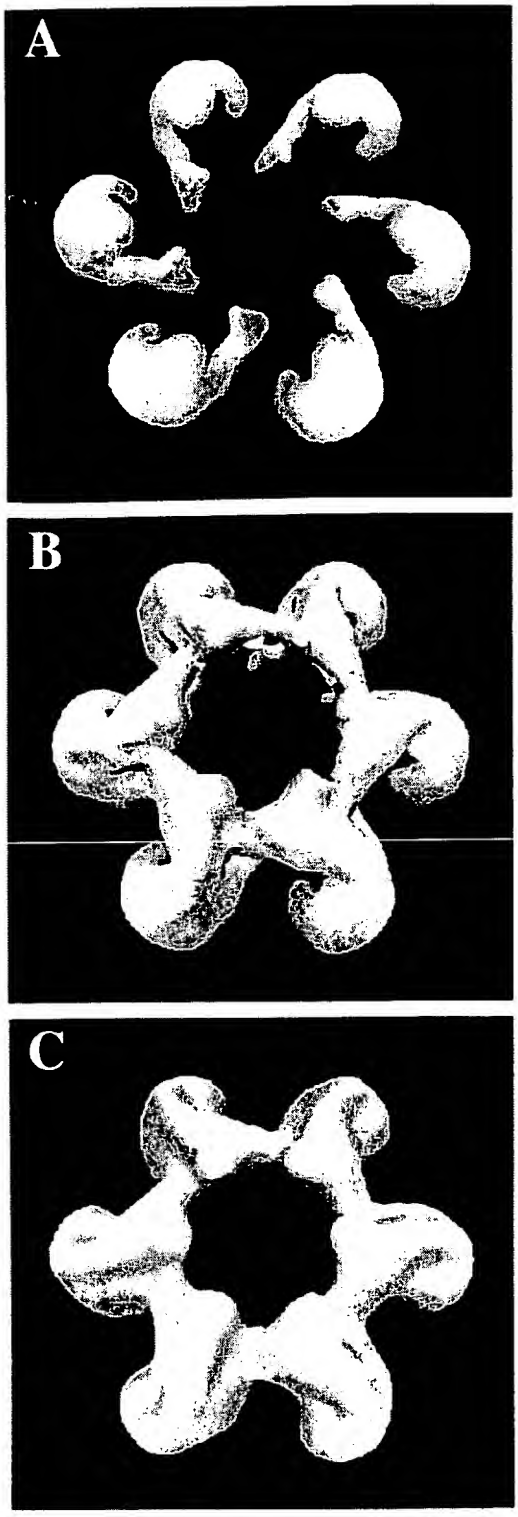

Figure 8. (a) 3D top view of the hexameric ring of P58 subunits, assembled *in silico* by use of reconstructed replicas. (b) Superimposition of a hexameric ring obtained by subtraction of the P58 ring from the holotoxin replicas onto the assembled ring of P58 fragments shown in (a). (c) 3D top view of WT VacA replicas.

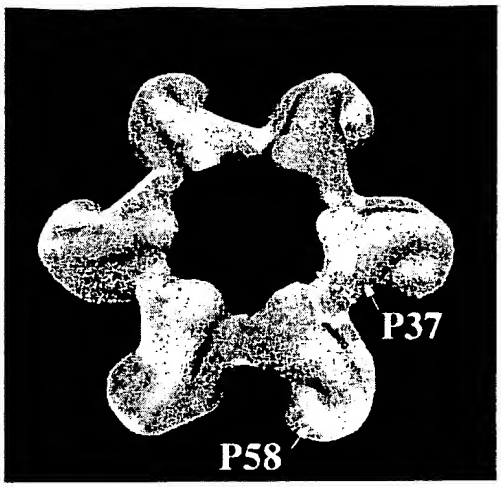

Figure 9. Model of the interaction of the 95 kDa monomer in the oligomeric structure. In the proposed model, alternate monomers in the oligomeric structure are shown in different colours in order to represent the interaction of the P37 subunit of one monomer with the P58 subunit of the adjacent monomer. P58, P58 subunit; P37, P37 subunit; dotted line, connecting loop between P58 and P37 subunit.

The P58 molecule is able to interact with the surface of target cells, thus strengthening the analogy with the B moiety transporter of the AB family of toxins. In contrast to the wild-type molecule, the P58 subdomain was not internalized by the target cell. This would suggest that either the 37 kDa subunit or the ability to form an oligomeric structure is required for cell entry. The mechanism of VacA entry into target cells is still somewhat obscure. Several data indicate that internalization is not through receptor mediated endocytosis. First, VacA requires pretreatment at pH < 5.0 for activity (de Bernard *et al.*, 1995). This would be unnecessary during normal endocytosis due to the lowering of pH in the endosomes. Furthermore, in contrast to endocytosis, internalization of VacA is very slow, taking several hours (Garner & Cover, 1996; Massari *et al.*, 1998). Nevertheless, it is clear that the P58 dimer has a sufficiently correct structure to permit at least a partial functional interaction with the cell.

Deletion of the central region of the p37 subunit abolished cytotoxic activity, showing that this region is necessary for cell intoxication and thus suggesting strongly that this domain carries the toxic activity of VacA. This is in good agreement with the report by de Bernard *et al.* (1998) showing that more than 250 amino acid residues can be removed from the carboxy terminus of VacA without any loss of intracellular activity. So far, no homology with any known protein was found in the P37 domain, suggesting a unique mode of action of VacA.

Taken together these data suggest that VacA has properties in common with the AB family of toxins. However, no catalytic activity has been described. Recent data suggest that VacA is capable of forming ion conductive channels in artificial membranes (Tombola *et al.*, 1999). In this regard, aerolysin, produced by *Aeromonas hydrophila*, causes vacuolation of the endoplasmic reticulum simply by forming pores in the plasma membrane of target cells (Abrami *et al.*, 1998). Members of the AB type toxin family possess both structural and functional similarity. However, despite such analogy, mechanisms of cell intoxication can be quite different. For example, ADP ribosylation in the case of cholera toxin or metalloprotease activity in the case of botulinum toxin. Furthermore, the way in which the B moiety inserts into the lipid bilayer can be very different (Cabiaux *et al.*, 1997).

Materials and Methods

Nucleotide sequence accession number

The nucleotide sequence of *vacA* from *H. pylori* SPM 326 has been deposited in the Genome Sequence Database (GSDB) under accession number AF 050318 (bp 1-117), AF 050395 (bp 1175-1614) and AF 050396 (bp 1971-2400). The coordinate of the nucleotide sequence has been assigned according to Atherton *et al.* (1995).

Construction of *H. pylori* SPM 326-P58

H. pylori SPM 326 that encodes a s1m1 type (Atherton *et al.*, 1995) *vacA* was chosen because of its relatively high transformability. This strain was transformed by the plasmid pEnKSF (Copass *et al.*, 1997) and a kanamycin-resistant sucrose-sensitive clone was selected as described (Copass *et al.*, 1997). The *H. pylori* SPM 326::pEnKSF was transformed by pRescue (see below) and a sucrose-resistant transformant coming from homologous recombination between the *vacA* fragment was selected as described (Copass *et al.*, 1997).

The pRescue was obtained in several steps. Primers JM3 (5'CCGCCGCTCGAGTGCAACAGAAATTTTCTAG TCTA3') and JM4 (5'ACTGGATAAAAGGGATC TGTATAA3') were used to amplify the chromosomal DNA of *H. pylori* CCUG 17834, corresponding to the region −72, +276. This 399 bp fragment was digested by *Eco*RI (an internal site) and *Xho*I, a site present in JM3 primer. The 348 bp fragment was gel-purified. The second fragment corresponds to a multiple cloning site that was obtained by hybridization of JM1 (5'AATTCGGATCCCTGCAGCCTCC3') and JM4 (5'TGG AGGCTGCAGGGATCCG3'), two oligonucleotides complementary and antiparallel. This 22 bp fragment was digested by *Eco*RI and *Eco*NI and ethanol purified. This multiple cloning site carries *Eco*RI, *Bam*HI, *Pst*I, and *Eco*NI. The last fragment was obtained by purifying the *Eco*NI/*Kpn*I fragment (1272 bp) of the pEK plasmid (Copass *et al.*, 1997).

The pRescue plasmid was obtained by cloning the *Xho*I/*Eco*RI, *Eco*RI/*Eco*NI, *Eco*NI/*Kpn*I fragment into the *Xho*I/*Kpn*I sites of pBSK+ (Short *et al.*, 1988). The sequence of this plasmid was checked using an automated sequencer. It contains the DNA coding for the signal sequence plus the 59 amino acid residues of the P37 subunit, connected in-frame with the P58 subunit *via* a multiple cloning site (MCS). This MCS can be used to fuse various pieces of DNA in order to generate various hybrid molecule with the P58 subunit.

Purification of P58

For the analysis of the expression, pelleted bacteria were resuspended in water, agitated and centrifuged. Supernatants of these water-extracts were used in immunoblot assays. The P58 molecule was purified from a culture supernatant of *H. pylori* SPM 326-P58 using the immuno-affinity chromatography system previously described by Reyrat *et al.* (1998).

NH_2-terminal sequencing

Automated sequence analysis of peptides was performed on a Beckman sequencer (LF 3000) equipped with an on-line phenylthiohydantoin-amino acid analyser system gold, according to the manufacturer.

Cross-linking experiments

Cross-linking experiments: 10 µl of P58 (95 µg/ml) were incubated three hours at room temperature with DMS (dimethylsuberimate) at a final concentration of 2 mg/ml in 0.2 M triethanolamine buffer (pH 8.5). After addition of the gel sample buffer, 0.25 of the samples were loaded on a SDS-9% PAGE. A similar amount of non-cross-linked P58 (2.5 µl) was used as a control to determine the amount of cross-linking. The gel was transferred onto a nitrocellulose membrane and the protein was revealed by immunoblot, using a polyclonal serum against the native molecule. For mass determination of the species in solution after cross-linking, 35 µl of P58 (95 µg/ml) were incubated as described above with DMS at a final concentration 2 mg/ml.

Maldi-tof mass spectrometry

Matrix-assisted laser desorbtion ionization-time of flight (Maldi-tof) mass spectra was acquired in a linear mode on a Brucker (Billerica, Mass.) reflex instrument equipped with a nitrogen laser (337 nm, 4 ns pulse). Samples were prepared by adding an aliquot of the protein in 1% (v/v) acetic acid to a saturated solution of sinapinic acid (3,5-dimethoxy-4-hydroxy-*trans*-cinnamic acid) (1:5 v/v). Approximately 1 pmol of protein in sinapinic acid was loaded onto the sample target for Maldi-tof ms. The air-dried sample matrix mixture was introduced into the mass spectrometer by means of a vacuum lock. Spectra were recorded by using an accelerated voltage of 28.5 kV. Spectra were calibrated by the analysis of a bovine serum albumin dimer (molecular mass, 132,861), which was used as an external calibrant.

Quick freeze, deep etching and electron microscopy

VacA molecules were prepared for microscopy by a procedure of absorption to Mica followed by freeze drying (Heuser, 1983). The samples were processed as described (Lupetti *et al.*, 1996).

Averaging and 3D reconstruction

Images were digitized by an Optronics Photoscan P1000 at a 25 µm sampling step. A gallery of about 1200

untitled images was collected. Images were aligned by a reference-free algorithm (Penczek *et al.*, 1992) or using handedness-lacking artificial references, and classified by correspondence analysis (Benzecri, 1992). A few clusters of homogeneous metal-covered replicas were selected and processed for random conical tilt reconstruction (Radermacher *et al.*, 1987) using corresponding views in tilted micrographs, as described by Lanzavecchia *et al.* (1998). A few 3D reconstructions were obtained from groups of about 200 images each one, with a resolution of 2.5 nm estimated by Fourier shell correlation criterion (Van Heel, 1987).

Cytotoxicity

To assay vacuolating activity, HeLa cells were cultured in plastic flasks in Dulbecco modified Eagle's medium (DMEM) containing 10% (v/v) fetal calf serum and 2 mM glutamine at 37°C in 5% (v/v) CO_2. The extent of vacuolation was determined quantitatively by measuring the uptake of neutral red as detailed previously (Cover & Blaser, 1992). Evaluation of the trans-epithelium resistance decrease induced on polarized MCDK cells was as described (Papini *et al.*, 1998, Pelicic *et al.*, (1999).

Expression of VacA P58 in HeLa cells

The gene encoding the VacA P58 toxin was cloned into an expression vector according to de Bernard *et al.* (1998). Briefly the sequence coding for amino acid residues 34 to 885 was amplified by PCR and cloned into plasmid pGEM7Zf(+) (Promega). PCR was carried out using, the specific synthetic oligonucleotides, P58f 5'CCGCGC**CTCGAG**ATGGCCTTTTTCACAACCGTGATCATTCCAGCC3' and P58r 5'GGGCCA**ATGCAT**TTAAGAACGTGCATTGCTAGTGGTGTT3', which were engineered in order to contain the start codon (5'ATG underlined) the stop codon (5'TTA underlined) and the recognition sequence for *Xho*I and *Nsi*I (bold), respectively. The PCR product was purified from agarose gel, digested by *Xho*I and *Nsi*I and subsequently cloned into the corresponding site of pGEM7Zf(+). This resulting plasmid (pGEMp58 J) was used to transfect HeLa cells as described (de Bernard *et al.*, 1998).

Binding of VacA and P58 to RK13 cells

Binding assays were performed as described (Pagliaccia *et al.*, 1998), but using the rabbit kidney cell line, RK13. Immune polyclonal IgGs raised against m1 VacA were used at a concentration of 15 µg/ml.

Assessment of cytotoxin binding and internalization by immunofluorescence microscopy

For assessment of binding, HeLa cells were incubated with P58 at 0°C for one hour, and processed with minor modifications as described (Garner & Cover, 1996). For cytotoxin internalization HeLa cells were incubated with either P58 or VacA at 37°C for three hours Cells were then fixed and membranes were solubilized as described (de Bernard *et al.*, 1997). Cells were probed with either anti-native VacA or mAb C1G9, in PBS and incubated with cells for one hour. After several washes with PBS, fluoresceinated anti-rabbit Ig were diluted 1:2000 in the same buffer and added for 30 minutes and then washed. Samples were mounted in 90% (v/v) glycerol, 0.2% (w/v) *N*-propylgallate in PBS and observed with a fluorescence microscope (Zeiss, Axioplan).

Source of anti-cytotoxin sera

Rabbit serum against m1 VacA has been described (Manetti *et al.*, 1995). Monoclonal antibody C1G9 has also been described (Reyrat *et al.*, 1998).

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Essential Role of a GXXXG Motif for Membrane Channel Formation by *Helicobacter pylori* Vacuolating Toxin*

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Helicobacter pylori **secretes a toxin, VacA, that can form anion-selective membrane channels. Within a unique amino-terminal hydrophobic region of VacA, there are three tandem GXXXG motifs (defined by glycines at positions 14, 18, 22, and 26), which are characteristic of transmembrane dimerization sequences. The goals of the current study were to investigate whether these GXXXG motifs are required for membrane channel formation and cytotoxicity and to clarify the role of membrane channel formation in the biological activity of VacA. Six different alanine substitution mutations (P9A, G13A, G14A, G18A, G22A, and G26A) were introduced into the unique hydrophobic region located near the amino terminus of VacA. The effects of these mutations were first analyzed using the TOXCAT system, which permits the study of transmembrane oligomerization of proteins in a natural membrane environment. None of the mutations altered the capacity of ToxR-VacA-maltose-binding protein fusion proteins to insert into a membrane, but G14A and G18A mutations markedly diminished the capacity of the fusion proteins to oligomerize. We then introduced the six alanine substitution mutations into the *vacA* chromosomal gene of *H. pylori* and analyzed the properties of purified mutant VacA proteins. VacA-G13A, VacA-G22A, and VacA-G26A induced vacuolation of HeLa cells, whereas VacA-P9A, VacA-G14A, and VacA-G18A did not. Subsequent experiments examined the capacity of each mutant toxin to form membrane channels. In a planar lipid bilayer assay, VacA proteins containing G13A, G22A, and G26A mutations formed anion-selective membrane channels, whereas VacA proteins containing P9A, G14A, and G18A mutations did not. Similarly, VacA-G13A, VacA-G22A, and VacA-G26A induced depolarization of HeLa cells, whereas VacA-P9A, VacA-G14A, and VacA-G18A did not. These data indicate that an intact proline residue and an intact $G^{14}XXXG^{18}$ motif within the amino-terminal hydrophobic region of VacA are essential for membrane channel formation, and they also provide strong evidence that membrane channel formation is essential for VacA cytotoxicity.**

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Helicobacter pylori is a Gram-negative bacterium that colonizes the gastric mucosa of humans (1). Colonization of the stomach by *H. pylori* is a strong risk factor for the development of peptic ulcer disease and distal gastric adenocarcinoma (2–4). Within the gastric mucosa, *H. pylori* organisms are found predominantly in the gastric mucus layer, but they also adhere to gastric epithelial cells (5).

Adherence of *H. pylori* to gastric epithelial cells triggers the initiation of processes that ultimately result in alteration of gastric epithelial cell function or gastric epithelial cell death. One of the important effector molecules used by *H. pylori* to modulate eukaryotic cell function is a secreted toxin known as VacA (6–8). The secreted VacA toxin is an 88-kDa protein, consisting of about 821 amino acids (9). These 88-kDa VacA monomers spontaneously self-assemble into large water-soluble flower-shaped oligomeric complexes, consisting predominantly of 12 or 14 monomeric subunits (10–12). When added to eukaryotic cells *in vitro*, the oligomeric form of VacA is nearly devoid of cytotoxic activity. However, exposure of oligomeric VacA to either acidic or alkaline pH results in disassembly of the oligomeric complexes into monomers that exhibit potent cytotoxic activity (10, 13–15).

The addition of VacA to eukaryotic cells causes multiple alterations in cell structure and function. When VacA is added to cells in the presence of weak bases, the most apparent effect is the development of cytoplasmic vacuolation (16, 17). VacA also induces permeabilization of epithelial monolayers (18), apoptosis (19–21), depolarization of the resting membrane potential (22, 23), and it interferes with the process of antigen presentation (24). After binding of VacA to the plasma membrane, the toxin is internalized by cells (15, 23, 25, 26). Based on experiments in which cells are transiently transfected with VacA-encoding plasmids (27, 28), it is presumed that VacA cytotoxicity results from toxin action at an intracellular site. Endosomal compartments and mitochondria have both been suggested as possible intracellular sites of VacA action (19, 29, 30).

Water-soluble VacA molecules are able to insert into lipid bilayers and form anion-selective membrane channels (22, 31–33). It has been hypothesized that VacA cytotoxicity results primarily from the formation of such channels. One model proposes that VacA forms anion-selective channels in the membranes of endosomes, which leads to osmotic swelling of these compartments (for review, see Refs. 8 and 34). However, it also has been hypothesized that VacA cytotoxicity may result primarily from an unrecognized enzymatic activity and that the formation of anion-selective membrane channels may be a secondary feature (for review, see Ref. 8). Support for the latter hypothesis is based on the recognition that numerous A-B type bacterial toxins exhibit an enzymatic activity and also exhibit a

capacity for membrane channel formation (for review, see Refs. 35 and 36).

In support of the view that VacA cytotoxicity results primarily from its capacity to form membrane channels, it has been reported that an inhibitor of anion-selective channels, NPPB,[1] blocks VacA cytotoxicity (22). In addition, we have reported that VacA-Δ(6–27), a VacA mutant containing a 22-amino acid deletion, lacks the capacity to form membrane channels and also lacks cytotoxic activity (37). Unfortunately, neither of these experimental approaches provides conclusive evidence indicating that membrane channel formation is essential for VacA cytotoxicity. Interpretation of experiments using NPPB is limited by the possibility that NPPB might exert multiple effects on cells or on the toxin rather than specifically inhibiting VacA channel activity. Also, conclusions from experiments with VacA-Δ(6–27) are limited by the possibility that a 22-amino acid deletion might alter multiple functional activities of the toxin rather than specifically ablating channel forming activity.

VacA contains a unique hydrophobic region located near its amino terminus, and it seems possible that such a region might be utilized for insertion of VacA into membranes during the process of membrane channel formation. In a previous study, we used a model system known as TOXCAT to analyze the functional role of the VacA hydrophobic region (38, 39). The results indicated that the VacA hydrophobic region (amino acids 1–32) mediated insertion of ToxR-VacA-maltose-binding protein (MBP) fusions into the inner membrane of *Escherichia coli* and also mediated protein dimerization. In contrast, a fusion protein containing a mutant VacA hydrophobic region (in which glycine 14 was replaced by alanine) inserted into the inner membrane but dimerized less efficiently than the fusion protein containing the wild-type VacA sequence. These experiments demonstrated that the VacA amino-terminal hydrophobic region is capable of inserting into membranes and undergoing dimerization.

Within the VacA hydrophobic region, there are three tandem G*XXX*G motifs, which are characteristic of transmembrane dimerization sequences (Fig. 1). In the current study, we sought to investigate the functional role of these three G*XXX*G motifs in membrane channel formation and cytotoxicity and to clarify the role of membrane channel formation in the biological activity of VacA. We report here that alanine substitution mutations at the Gly-14 and Gly-18 positions abolish the capacity of VacA to form membrane channels and also abolish the capacity of VacA to induce cytotoxic effects. We also report that the $G^{14}XXXG^{18}$ motif is required for dimerization of VacA in a membrane using the TOXCAT system. These results provide further evidence that VacA cytotoxicity occurs via a process that requires membrane channel formation and indicate that the amino-terminal hydrophobic region of VacA plays an essential role in both membrane channel formation and cytotoxicity.

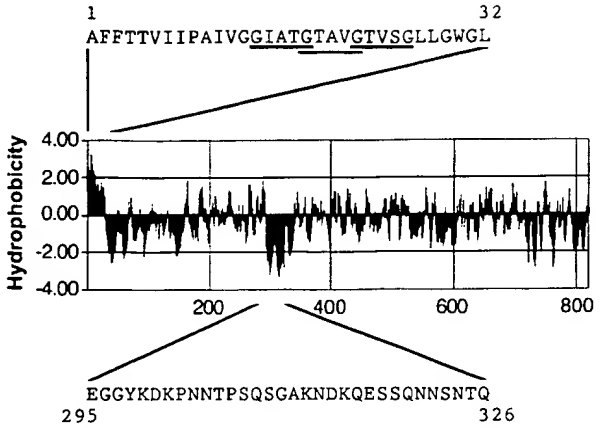

FIG. 1. **Hydrophobicity plot of the mature secreted VacA toxin from *H. pylori* 60190 (79).** The predicted hydrophobicity of the mature VacA toxin from *H. pylori* 60190 was analyzed by the method of Kyte and Doolittle (80). *Capital letters* indicate the amino-terminal sequence of VacA as well as a hydrophilic region of VacA which is susceptible to proteolytic cleavage. The *underlined* segments represent three tandem G*XXX*G motifs, characteristic of transmembrane oligomerization regions, located near the amino terminus of VacA.

MATERIALS AND METHODS

Analysis of VacA Using the TOXCAT System—The TOXCAT system (Fig. 2) was originally developed by Russ and Engelman (39) to study transmembrane helix-helix associations in a natural membrane environment. In this system, a putative transmembrane (TM) sequence is cloned between a sequence encoding the transcription activator domain of *Vibrio cholerae* ToxR and a sequence encoding the periplasmic domain of the *E. coli* MBP. To determine whether the putative transmembrane sequence mediates transmembrane oligomerization, the ToxR-TM-MBP fusion proteins are expressed in *E. coli*. Membrane localization of the fusion protein is detected based on complementation of a nonpolar *malE* mutant *E. coli* strain. Insertion of the ToxR-TM-MBP fusion protein into the inner membrane, such that the MBP domain localizes to the periplasmic space, is detected by determining whether the bacteria are able to transport maltose and thus grow on maltose-minimal medium. Dimerization of the fusion protein is determined based on expression of the *cat* gene, which is under the control of the dimerization-dependent transcription activator ToxR. *E. coli* strains expressing ToxR-TM-MBP fusion proteins that dimerize are resistant to chloramphenicol, whereas strains expressing fusion proteins that lack a dimerization sequence are sensitive to chloramphenicol. Plasmid pccVacA-wt and pccVacA-G14A have been described previously (38). Additional plasmids containing mutations within the *vacA* amino-terminal sequence were constructed using the method of Perrin and Gilliland (40). As a control, the portion of *vacA* corresponding to amino acids 295–326 was also cloned into the TOXCAT plasmid, generating pccVacA-295/326. Chloramphenicol acetyltransferase (CAT) enzyme was measured by an antigen-capture enzyme-linked immunosorbent assay (Roche Molecular Biochemicals) according to the manufacturer's instructions.

Construction of H. pylori Strains Expressing Mutant VacA Proteins—Plasmid pAV202 (41), which contains 567 bp of *cysS*, the *cysS-vacA* intergenic region, 883 bp of the *vacA* coding sequence from *H. pylori* 60190, and a CAT gene inserted at the 3′-terminus of *cysS*, was used as the template for all site-directed mutagenesis reactions. Mutations encoding single amino acid substitutions were introduced into pAV202 using the method of Perrin and Gilliland (40) or inverse PCR (42). These mutations then were introduced into the *H. pylori* chromosomal *vacA* gene by natural transformation and allelic exchange, as described previously (37, 41, 43). Mutants were selected on *Brucella* agar plates containing 5 μg/ml chloramphenicol. To confirm that the desired mutations had been introduced successfully into the chromosomal *vacA* gene, fragments of the 5′-end of *vacA* were PCR amplified and analyzed by restriction endonuclease digestion and nucleotide sequence analysis.

Purification of H. pylori VacA—H. pylori strains were grown in sulfite-free *Brucella* broth containing activated charcoal (44). VacA was purified in an oligomeric form from culture supernatants of *H. pylori* strains, as described previously (10). Purified VacA preparations were routinely acid activated before the addition of VacA to planar lipid bilayer chambers or cell culture wells, as described previously (13, 15).

Planar Lipid Bilayer Methodology—Planar lipid bilayers, composed of egg phosphatidylcholine/dioleoylphosphatidylserine/cholesterol (55:15:30 mol%) dissolved in *n*-decane were prepared as described previously (31, 33, 37, 41). Purified acid-activated VacA toxins were added to the lipid bilayers in a buffer consisting of 5 mM citric acid (pH 4.0) and 2 mM EDTA, with the salt composition as described under "Results." Membrane currents were measured as described previously (37, 41). The potential is indicated relative to the cis side, defined as the chamber to which the protein was added. Permeability ratios were determined from the Goldman-Hodgkin-Katz equation (45), after the membrane voltage for zero current (reversal potential) in asymmetric salt

[1] The abbreviations used are: NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid; CAT, chloramphenicol acetyltransferase; MBP, maltose-binding protein; TM, transmembrane; wt, wild-type.

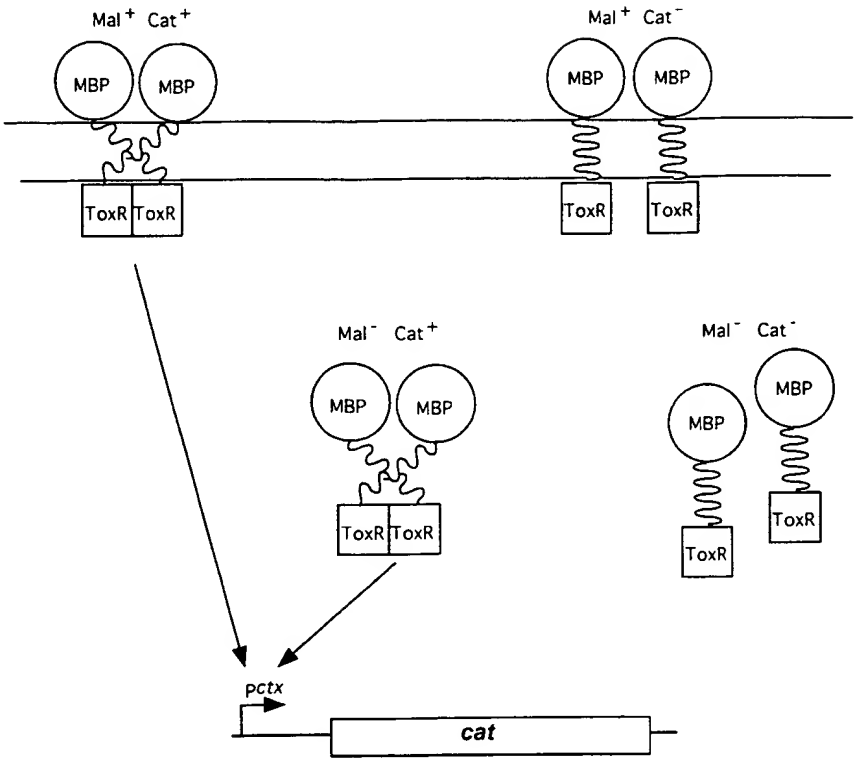

FIG. 2. **Schematic illustration of the principles on which the TOXCAT assay is based (39).** VacA sequences (depicted as *coiled lines*) were cloned between a sequence encoding the transcription activator domain of *V. cholerae* ToxR (*squares*) and a sequence encoding the periplasmic domain of the *E. coli* MBP (*circles*) so that ToxR-VacA-MBP fusion proteins would be expressed. If ToxR-VacA-MBP fusion proteins insert into the *E. coli* inner membrane such that the MBP domain localizes to the periplasmic space, cells are able to transport maltose and thus can grow in maltose-minimal medium (designated *Mal*$^+$). Dimerization of the fusion proteins results in the activation of the dimerization-dependent transcription activator ToxR, which leads to expression of the *cat* gene from the ToxR-activated cholera toxin promoter (*pctx*). Cells expressing *cat* are designated *Cat*$^+$. The figure depicts four possible phenotypes expressed in the TOXCAT assay.

concentrations was measured. Statistical significance was analyzed using Student's *t* test.

Analysis of Membrane Potential of HeLa Cells—Experiments to analyze the membrane potential of HeLa cells were performed as described previously (22, 23). Briefly, HeLa cells were detached with trypsin/EDTA, washed, and then incubated with bis-(3-propyl-5-oxoisoxazol-4-yl)pentamethine oxonol (oxonol VI) (Molecular Probes) in a final concentration of 2.5 μM for 15 min at 37 °C. A cell suspension (2 ml) was placed in a stirred quartz cuvette at 37 °C in a PerkinElmer Life Sciences LS50B fluorometer. After stabilization of the fluorescence signal (excitation, 585 nm; emission, 645 nm), acid-activated VacA toxins (final concentration 5 μg/ml) were added to the cells, and changes in the fluorescence were monitored. Depolarization causes a potential-dependent change in the cytoplasmic/transmembrane distribution of the fluorophore, which is accompanied by a change in fluorescence (46).

Cell Culture—HeLa cells were grown in minimal essential medium (modified Eagle's medium containing Earle's salts) containing 10% fetal bovine serum. Acid-activated VacA preparations were incubated with cultured cells in a microtiter format, as described previously (13, 15). After incubation for 24 h, the cells were examined by inverted light microscopy. Samples that induced vacuolation in >50% of cells were scored as positive for the vacuolating cytotoxin phenotype. In some experiments, vacuolation was also quantified by neutral red uptake assay (47).

RESULTS

Identification of VacA Amino Acids Required for Transmembrane Protein Dimerization in a TOXCAT Model System—The amino-terminal hydrophobic segment of VacA contains three tandem GXXXG motifs, which are characteristic of transmembrane dimerization sequences (Fig. 1). To investigate the role of these GXXXG motifs in greater detail, we used the TOXCAT model system (Fig. 2), which has been used to study transmembrane oligomerization of proteins in a natural membrane environment (38, 39, 48–50). We used a previously described plasmid designated pccVacA-wt (38), which encodes a fusion protein in which the amino-terminal 32 amino acids of VacA are inserted between the transcription activation domain of ToxR and the periplasmic domain of MBP, and which also contains the *cat* gene under the control of the *V. cholerae ctx* promoter. To examine the role of the GXXXG motifs, we used four related plasmids that were identical to pccVacA-wt except for the presence of mutations within the *vacA* sequence which resulted in substitution of the Gly-14, Gly-18, Gly-22, and Gly-26 residues by alanine. We also constructed two related plasmids containing mutations within the *vacA* sequence which resulted in substitution of the Pro-9 and Gly-13 residues by alanine. Pro-9 was selected for analysis because this residue is reported to be important for VacA cytotoxic activity (51). Gly-13 was selected for analysis as a control because this glycine residue is not located within a GXXXG motif. As an additional control, a 32-amino acid segment (amino acids 295–326) from a predicted hydrophilic region of VacA (Fig. 1) was also cloned into the TOXCAT vector. When analyzed by immunoblot analysis using anti-MBP antiserum, each of these plasmid-containing *E. coli* strains produced ToxR-TM-MBP fusion proteins (Fig. 3*A*).

To determine whether the putative transmembrane sequences encoded by these plasmids promoted membrane localization of the ToxR-TM-MBP fusion proteins, *E. coli* strains containing these plasmids were cultured on M9-maltose medium. Consistent with previous results (38), *E. coli* strain MM39 containing pccVacA-wt was able to grow on M9-maltose medium, whereas MM39 with no plasmid was unable to grow on this medium (Fig. 3*B*, data not shown). As expected, *E. coli* containing pccVacA-295/326 was unable to grow on this medium. The failure of this strain to grow on M9-maltose medium is consistent with the failure of this VacA hydrophilic segment to mediate insertion of the TOXCAT fusion protein into the inner membrane of *E. coli*. Each of the plasmids containing mutant *vacA* sequences grew on M9-maltose medium, similar to *E. coli* containing the parental wild-type *vacA* plasmid (Fig. 3*B*). These results indicate that none of the single amino acid substitutions in the VacA hydrophobic region altered the capacity of this VacA segment to mediate insertion of the ToxR-TM-MBP fusion proteins into a membrane.

We next analyzed whether the mutant VacA segments were able to mediate dimerization of the TOXCAT fusion proteins. As reported previously (38), *E. coli* MM39 containing pccVacA-wt produced high levels of CAT, whereas *E. coli* MM39

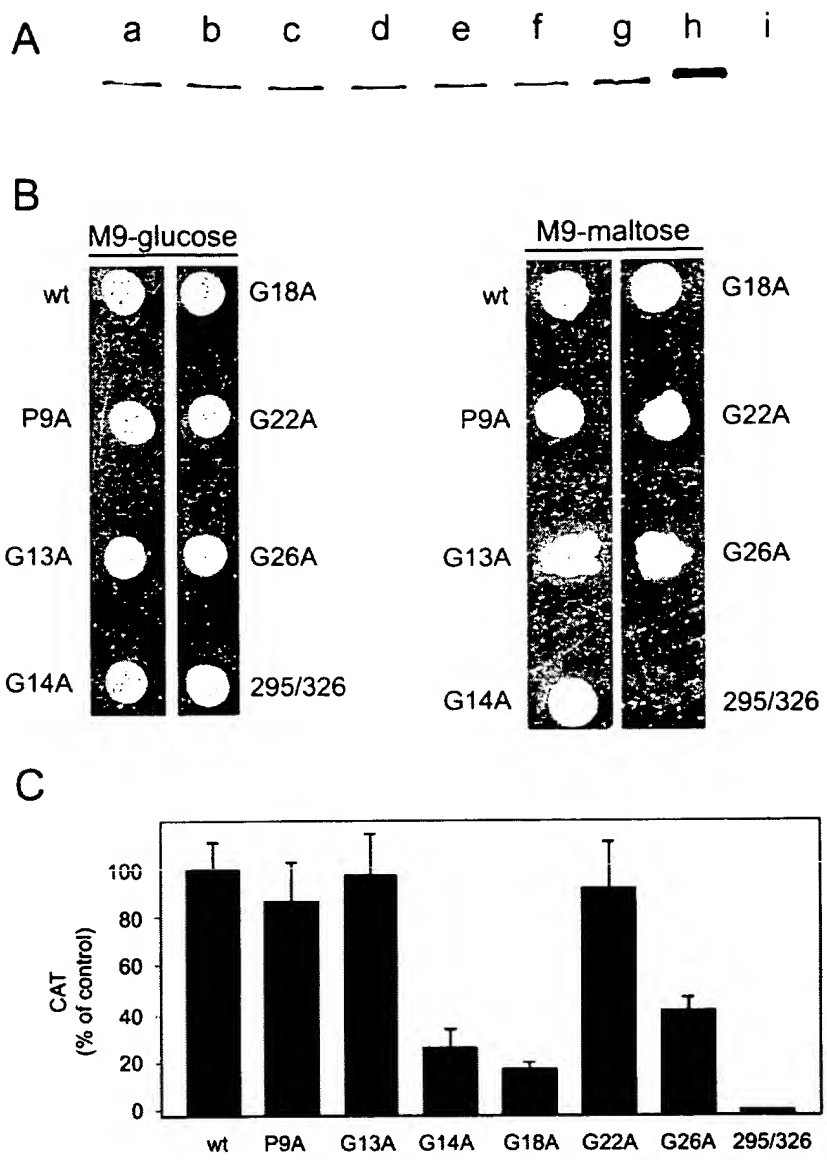

FIG. 3. **Analysis of mutations in the VacA hydrophobic region using the TOXCAT system (39).** *A*, expression of ToxR-VacA-MBP fusion proteins. *E. coli* strain MM39 carrying plasmid pccVacA-wt (*lane a*), pccVacA-P9A (*lane b*), pccVacA-G13A (*lane c*), pccVacA-G14A (*lane d*), pccVacA-G18A (*lane e*), pccVacA-G22A (*lane f*), pccVacA-G26A (*lane g*), pccVacA-295/326 (*lane h*), and no plasmid (*lane i*) were cultured in Luria-Bertani medium to an A_{600} of about 0.3. Equal culture volumes were pelleted, lysed in SDS-sample buffer, separated by SDS-PAGE, and immunoblotted using anti-MBP antiserum (New England Biolabs). *B*, membrane insertion of ToxR-VacA-MBP fusion proteins determined by growth on M9-maltose. *E. coli* strain MM39 carrying plasmid pccVacA-wt, pccVacA-P9A, pccVacA-G13A, pccVacA-G14A, pccVacA-G18A, pccVacA-G22A, pccVacA-G26A, and pccVacA-295/326 were grown on M9-glucose plates. Bacteria were resuspended in M9-maltose broth at an A_{600} of ~0.3. Strains were then spotted onto either M9-glucose or M9-maltose plates. All strains, except for MM39 pccVacA-295/326, grew on M9-maltose, indicating that the fusion proteins inserted into the *E. coli* inner membrane. *C*, expression of CAT by *E. coli* strains. *E. coli* strains were cultured in Luria-Bertani medium to an A_{600} of about 0.3. CAT protein levels from each strain was quantified using a CAT enzyme-linked immunosorbent assay. Results represent the mean ± S.D. from triplicate cultures. MM39 pccVacA-G14A, pccVacA-G18A, pccVacA-G26A, and pccVacA-295/326 produced significantly less CAT than did MM39 pccVacA-wt (Student's *t* test; $p < 5 \times 10^{-7}$).

containing no plasmid produced no CAT enzyme (Fig. 3*C* and data not shown). The control strain containing pccVacA-295/326 produced barely any detectable CAT. Compared with the *E. coli* MM39 containing pccVacA-wt, *E. coli* containing plasmids encoding P9A, G13A, and G22A mutations expressed similar high levels of CAT enzyme. In contrast, *E. coli* containing plasmids encoding G14A, G18A, or G26A mutations expressed significantly lower levels of CAT than did the strain containing pccVacA-wt. These results indicate that several glycine residues within the VacA hydrophobic region contribute to transmembrane protein dimerization and that the glycines located within the $G^{14}XXXG^{18}$ motif are particularly important for protein dimerization in this system.

Role of the VacA Amino-terminal Hydrophobic Region in Vacuolating Cytotoxic Activity—Although the experiments described above using the TOXCAT system provide useful insights into the function of the VacA amino-terminal segment, it seems possible that the conformation and function of this segment might be considerably different in the context of the native VacA protein interacting with eukaryotic cells, rather than the TOXCAT fusion proteins interacting with the inner membrane of *E. coli*. Therefore, we next undertook studies designed to analyze the function of the amino-terminal hydrophobic region in the context of the intact VacA protein produced by *H. pylori*.

For these studies, we introduced mutations encoding P9A, G13A, G14A, G18A, G22A, and G26A into the *H. pylori* chromosomal *vacA* gene, as described under "Materials and Methods." Each of the mutant strains expressed and secreted VacA proteins of the expected size (Fig. 4*A*). The level of VacA produced by each of these mutant strains was lower than the level of VacA produced by a wild-type *H. pylori* strain but similar to the level of VacA produced by a control strain without any *vacA* mutations in which a chloramphenicol resistance cassette was introduced upstream from the *vacA* promoter, within the *cysS-vacA* intergenic region (data not shown). This indicates that the presence of the chloramphenicol cassette in this region results in diminished levels of VacA expression, presumably by altering levels of *vacA* transcription (52). All of the mutant VacA proteins were detected as large oligomeric structures when *H. pylori* culture supernatants were fractionated by gel filtration chromatography. Thus, although TOXCAT experiments indicated an apparent role for Gly-14 and Gly-18 in dimerization within a membrane, these residues were not essential for oligomerization of the intact VacA proteins in solution. It seems likely that oligomerization of water-soluble VacA monomers

involves interactions of multiple domains rather than only homotypic interactions of the amino-terminal hydrophobic regions of different monomers. In addition, there may be substantial differences between the structure of VacA in solution compared with the structure of VacA in a membrane.

Next, we sought to determine the effect of the various mutations described above on the capacity of VacA to induce vacuolating cytotoxic effects on eukaryotic cells. Purified preparations of each of the VacA proteins were standardized by protein concentration, acid-activated, and then added to HeLa cells. As a control, wild-type VacA was purified from the wild-type *H. pylori* strain 60190. VacA proteins containing G13A, G22A, or G26A mutations each induced vacuolation of HeLa cells (Table I). However, quantification of VacA-induced vacuolation by neutral red uptake assay (47) revealed that VacA-G13A and VacA-G22A were consistently less active than wild-type toxin from *H. pylori* strain 60190 (Fig. 4*B*). In contrast to VacA-G13A, VacA-G22A, and VacA-G26A, VacA proteins containing P9A, G14A, or G18A mutations did not induce any detectable vacuolation, even at the highest protein concentration tested (Table I and Fig. 4*B*). These data indicate that the vacuolating activity of VacA is detectably altered by several of the alanine substitution mutations. However, an intact proline and an intact $G^{14}XXXG^{18}$ motif are absolutely essential for vacuolating activity.

TABLE I
Analysis of VacA channel forming properties and cytotoxic activity

VacA sample	Cytotoxic activity[a]	Time to −100 pA[b]	Ion selectivity ratio[c] (P_{Cl}/P_{Na})
		min	
Wild-type VacA	+	44 ± 25 (n = 9)	4.9 ± 0.9 (n = 9)
P9A	−	>700 (n = 7)[d]	ND[e]
G13A	+	52 ± 34 (n = 5)	5.0 ± 0.4 (n = 5)
G14A	−	>760 (n = 3)[d]	ND[e]
G18A	−	>844 (n = 4)[d]	ND[e]
G22A	+	80 ± 45 (n = 9)	4.2 ± 0.4 (n = 9)
G26A	+	49 ± 17 (n = 5)	4.9 ± 0.7 (n = 5)

[a] Analysis of VacA cytotoxicity was conducted by incubating purified acid-activated VacA proteins (20 μg/ml) with HeLa cell monolayers. Proteins that induced vacuolation in >50% of cells were scored as positive for the vacuolating cytotoxic phenotype. Results were confirmed by neutral red uptake assay (Fig. 4*B*).

[b] VacA preparations (30 nM, ~2.7 μg/ml) were added to planar lipid bilayers composed of egg phosphatidylcholine/dioleoylphosphatidylserine/cholesterol (55:15:30 mol%) in a buffer containing 5 mM citric acid, 100 mM sodium chloride, and 2 mM EDTA (pH 4). The time required to produce a current of 100 pA at −50 mV was then determined. Results represent the mean ± S.D. from multiple independent determinations for each sample tested. n = the number of successfully completed experiments for each sample.

[c] Reversal potential was measured in *cis:* 200 mM NaCl, *trans:* 100 mM NaCl using 30 nM concentrations of VacA samples. The number of experiments is designated (n). Results represent mean ± S.D.

[d] Each of these samples was tested in at least 10 different experiments. When testing these samples, the membrane frequently ruptured before the detection of any current (data not shown). The mean time to membrane rupture was 450 min. Results from experiments in which the membrane ruptured were excluded from our analysis.

[e] ND indicates not determined.

Role of the VacA Amino-terminal Hydrophobic Region in Formation of Channels in Planar Lipid Bilayers—To test the hypothesis that the $G^{14}XXXG^{18}$ motif or perhaps one of the other GXXXG motifs contributes to the capacity of VacA to form anion-selective membrane channels, VacA proteins containing the mutations described above were purified from *H. pylori* broth culture supernatant. These purified proteins then were standardized according to protein concentration and were tested for the capacity to form membrane channels in a planar lipid bilayer system (37, 41). When tested at a VacA protein concentration of 30 nM, wild-type VacA formed anion-selective membrane channels in the expected manner, as described previously (Table I). VacA proteins containing G13A, G22A, or G26A mutations also formed membrane channels, with an anion selectivity similar to that of channels formed by wild-type VacA. The rate of membrane channel formation was similar among these toxins, except for a slightly slower rate of membrane channel formation by VacA-G22A. In contrast, formation of membrane channels by VacA proteins containing P9A, G14A, or G18A mutations could not be detected under these conditions (Table I). These results indicate that Pro-9, Gly-14, and Gly-18 are essential for channel formation by VacA.

Role of the VacA Amino-terminal Hydrophobic Region in Depolarization of HeLa Cells—When added to HeLa cells, VacA

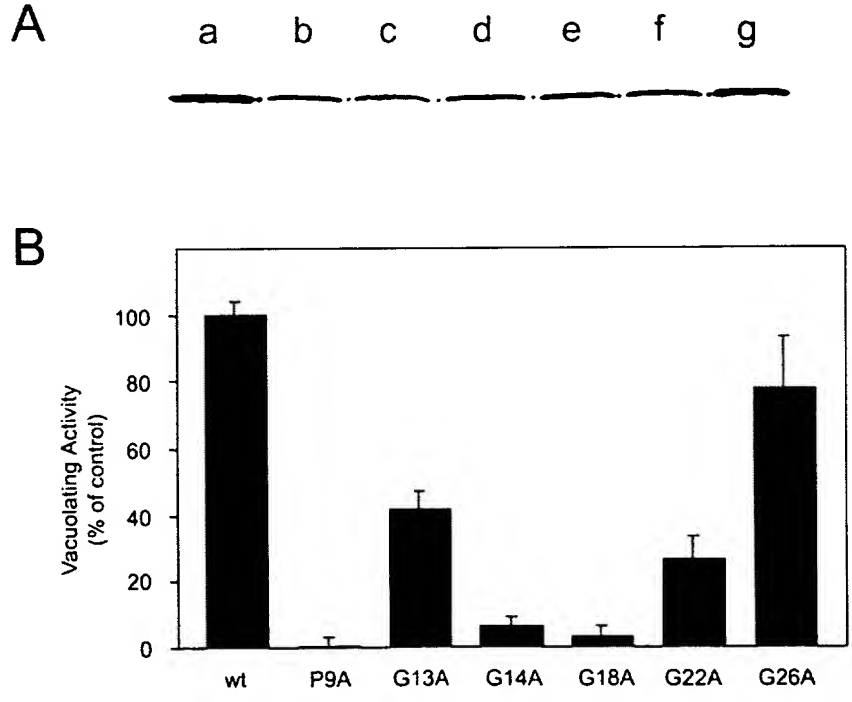

FIG. 4. **Expression and vacuolating activity of wild-type and mutant VacA proteins secreted by *H. pylori*.** *A*, expression of VacA proteins. *H. pylori* mutant strains were grown in broth culture, and broth culture supernatants were analyzed by immunoblot assay using anti-VacA serum. *Lane a*, *H. pylori* 60190 expressing wild-type VacA; *b*, VacA-P9A; *c*, VacA-G13A; *d*, VacA-G14A; *e*, VacA-G18A; *f*, VacA-G22A; *g*, VacA-G26A. Each of the strains expressed and secreted a VacA protein. *B*, vacuolating activity. Equal amounts (20 μg/ml) of purified VacA proteins were added to the medium overlying HeLa cells. Vacuolating activity was measured by neutral red uptake assay (47). Results represent the means ± S.D. from duplicate experiments, each performed in triplicate, and are expressed as the percent of neutral red uptake relative to control cells treated with wild-type VacA purified from *H. pylori* strain 60190.

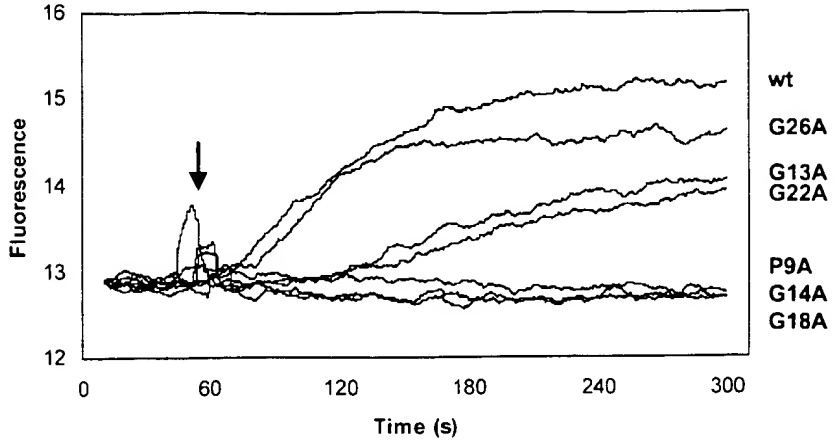

FIG. 5. **Analysis of membrane depolarization induced by mutant VacA proteins.** VacA proteins were purified from *H. pylori* broth culture supernatants as described under "Materials and Methods." HeLa cells were loaded with oxonol VI (a probe used to monitor membrane potential). After the addition of 5 μg/ml acid-activated VacA proteins, changes in fluorescence were monitored. The *arrow* indicates the time at which toxin was added to the cuvette. Wild-type VacA, VacA-G26A, VacA-G13A, and VacA-G22A induced membrane depolarization, whereas VacA-P9A, VacA-G14A, and VacA-G18A did not.

forms channels in the plasma membrane, which results in depolarization of the resting membrane potential (22, 23). To analyze further the role of the VacA amino-terminal hydrophobic region, we tested the capacity of each of the VacA preparations described above to induce depolarization of HeLa cells. As expected, wild-type VacA induced membrane depolarization in a manner similar to that described previously (Fig. 5). VacA proteins containing G13A, G22A, or G26A mutations also induced depolarization of the resting membrane potential. In contrast, no depolarization was induced by VacA proteins containing P9A, G14A, or G18A mutations. Thus, P9A, G14A, and G18A mutations resulted in loss of the capacity to form membrane channels when analyzed in the planar lipid bilayer system (Table I) as well as loss of the capacity to depolarize HeLa cells (Fig. 5).

DISCUSSION

Several previous studies have shown that amino acid sequences near the amino terminus of VacA play an important role in the process by which VacA exerts cytotoxic effects. The experimental system used in most of these previous studies has involved transiently transfecting HeLa cells with plasmids encoding VacA (27, 28, 37, 51, 53–55). When analyzed in this manner, intracellular expression of wild-type VacA induces cell vacuolation, whereas intracellular expression of truncated forms of VacA which lack as few as 10 amino acids at the amino terminus fails to induce cell vacuolation (28, 53). To identify individual amino acids that might be important for cytotoxic activity, alanine scanning mutagenesis of VacA residues 6–17 has been undertaken (51). When transfected into HeLa cells, plasmids encoding VacA-P9A and VacA-G14A fail to induce intracellular vacuolation, whereas plasmids encoding other single alanine mutations induce cell vacuolation in a manner indistinguishable from wild-type VacA (51). Additional studies indicating the importance of amino acid sequences near the amino terminus of VacA have used allelic exchange mutagenesis of the chromosomally encoded *vacA* gene in *H. pylori* to produce mutant toxins (37, 41, 43). When analyzed in this manner, VacA toxins in which a portion of the amino-terminal region was deleted (VacA-(Δ6–27)) or in which a 12-amino acid extension was added to the amino terminus (s2/m1 VacA) exhibit defects in vacuolating activity (37, 41).

In the current study, we investigated further the functional role of the VacA amino terminus by analyzing a panel of mutant *H. pylori* toxins. In one or more assays of VacA function, each of the mutant toxins examined exhibited at least a partial diminution in activity compared with wild-type VacA. However, the most dramatic and consistent defects were observed with VacA-P9A, VacA-G14A, and VacA-G18A. Our data confirm results from transient transfection experiments which indicated that P9A and G14A mutations ablate cytotoxic activity (51), and they indicate that a G18A mutation also ablates cytotoxic activity. The essential role played by Pro-9, Gly-14, and Gly-18 in VacA cytotoxic activity thus helps to explain the defect in cytotoxic activity observed in the previously characterized mutant toxin VacA-(Δ6–27) (37).

It has been proposed that the cytotoxicity of VacA is dependent on the capacity of this toxin to form anion-selective membrane channels (22, 31–33, 37, 41). In the present study, we found that the three nontoxic mutant VacA proteins (VacA-P9A, VacA-G14A, and VacA-G18A) were each defective in membrane channel formation, whereas the other mutant VacA proteins retained the capacity for both cytoplasmic vacuolation and membrane channel formation. The results obtained in a planar lipid bilayer assay were similar to those obtained in a HeLa cell depolarization assay. Thus, the current data provide further support for the hypothesis that VacA channel forming activity is essential for the formation of intracellular vacuoles. However, the current data do not exclude the possibility that VacA may have other biological activities in addition to the capacity for channel formation.

Studies of the mutant toxin VacA-Δ(6–27) indicated that the VacA amino-terminal hydrophobic region does not play a role in toxin binding to HeLa cells or toxin internalization by cells (37), but they suggested that the amino-terminal hydrophobic region plays a role in membrane channel formation. The analysis of mutant toxins containing single alanine substitutions in the current study now provides strong evidence that the amino-terminal hydrophobic region plays an essential role in membrane channel formation. It may be presumed that the VacA amino-terminal hydrophobic region either forms part of the channel or plays an indirect role by stabilizing the structure of the channel. In support of the former possibility, analysis of the predicted hydrophobicity of VacA suggests that the amino-terminal segment is the only contiguous stretch of hydrophobic amino acids long enough to span a membrane, and results obtained using the TOXCAT system indicate that this amino-terminal hydrophobic region is indeed capable of insertion into a membrane (Ref. 38 and the present study). It is not uncommon for the pores of ion channels to be lined with hydrophobic amino acids (56–58). However, it is possible that, in addition to the amino-terminal hydrophobic region, other VacA sequences may also insert into the membrane to form a functional channel. In support of this hypothesis, results of several studies have suggested that multiple regions of the VacA protein are capable of inserting into lipid membranes (59–61).

The appearance of VacA oligomers associated with membranes, as well as the kinetics of membrane channel formation by VacA, suggest that VacA channels are hexameric structures

(10, 12, 31, 33). It seems likely that multiple VacA-VacA interactions contribute to the formation of VacA membrane channels. Results obtained using the TOXCAT system indicate that the VacA amino-terminal hydrophobic region is capable of mediating dimerization within a membrane environment (Ref. 38 and the present work). In addition, previous studies have provided evidence indicating the occurrence of interactions between other domains of the toxin (54, 55, 62). The existence of multiple VacA-VacA interaction domains may explain why deletion of the amino-terminal hydrophobic region does not disrupt the formation of VacA oligomeric structures in solution (37).

VacA sequences containing G14A and G18A mutations exhibited defects in the TOXCAT assay of protein dimerization, whereas the P9A mutation did not alter the activity of the VacA sequence in this assay. This suggests that the P9A mutation alters VacA function in a manner different from the effects conferred by the G14A and G18A mutations. Proline residues are known to be associated with turns or bends in secondary structure, and therefore we speculate that the P9A mutation alters the secondary structure of VacA within this region. GXXXG motifs (such as that formed by Gly-14 and Gly-18) are known to be important in the ability of transmembrane α-helices to form homo-oligomers within a membrane environment (38, 39, 48–50). The significance of the GXXXG motif has been most widely studied using glycophorin A as a model transmembrane protein (39, 63–66). In addition, the importance of GXXXG motifs has been reported in a variety of other transmembrane proteins (49, 67–69). Recently, the GXXXG motif has been identified as a motif involved in helix-helix interactions in soluble proteins as well (70). Thus, the inactivity of VacA sequences containing G14A and G18A mutations in the TOXCAT system is consistent with the established role of GXXXG motifs in protein-protein interactions.

The structure of the membrane insertion domain is known for only a few bacterial toxins. In the case of toxins such as colicins and the δ-endotoxins of *Bacillus thuringiensis*, the membrane insertion domain consists of a bundle of hydrophobic and amphipathic α-helices (71, 72), and in the case of toxins such as *Staphylococcus aureus* α-toxin and *Clostridium perfringens* perfringolysin O, the membrane insertion domain consists of a β-barrel (73, 74). Like VacA, the α-toxin from *S. aureus* also contains three tandem copies of the motif GXXXG (75). However, unlike the VacA amino-terminal hydrophobic region, the glycine-rich region of α-toxin is amphipathic. Structural analyses have indicated that this glycine-rich or "hinge" region of α-toxin inserts into the membrane of sensitive cells to form a transmembrane β-barrel (76–78). Thus, although GXXXG motifs are frequently associated with transmembrane α-helices (48), these motifs occasionally may be found within transmembrane β-barrels (76–78). In future investigations, it will be important to correlate the functional studies of the VacA amino-terminal hydrophobic region described in this study with high resolution structural analysis of VacA membrane channels.

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